30713

NATIONAL LIBRARY
OTTAWA

CANADA

BIBLIOTHÈQUE NATIONALE
OTTAWA

NAME OF AUTHOR... Shelly L. Hook .....

TITLE OF THESIS... DISTRIBUTION OF CELL SURFACE RECEPTOR SITES FOR CONCANAVALIN A IN THE EARLY CHICK EMBRYO .....

UNIVERSITY..... UNIVERSITY OF ALBERTA .....

DEGREE FOR WHICH THESIS WAS PRESENTED..... M. Sc .....

YEAR THIS DEGREE GRANTED..... 1976 .....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed)..... Shelly Hook .....

PERMANENT ADDRESS:

Dept. of Physiology
University of Alberta
Edmonton, Alberta

DATED..... July 21 ..... 19 76

NL-91 (10-68)

INFORMATION TO USERS

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

This copy was produced from a microfiche copy of the original document. The quality of the copy is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Canadian Theses Division
Cataloguing Branch
National Library of Canada
Ottawa, Canada K1A 0N4

AVIS AUX USAGERS

LA THESE A ETE MICROFILMEE TELLE QUE NOUS L'AVONS RECUE

Cette copie a été faite à partir d'une microfiche du document original. La qualité de la copie dépend grandement de la qualité de la thèse soumise pour le microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

NOTA BENE: La qualité d'impression de certaines pages peut laisser à désirer. Microfilmee telle que nous l'avons reçue.

Division des thèses canadiennes
Direction du catalogage
Bibliothèque nationale du Canada
Ottawa, Canada K1A 0N4

THE UNIVERSITY OF ALBERTA

DISTRIBUTION OF CELL SURFACE RECEPTOR SITES FOR CONCANAVALIN A IN THE EARLY CHICK EMBRYO

by

© SHELLY L. HOOK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

CELL BIOLOGY

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

FALL, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled DISTRIBUTION OF CELL SURFACE RECEPTOR SITES FOR CONCANAVALIN A IN THE EARLY CHICK EMBRYO submitted by SHELLY L. HOOK in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

E. J. Sander
Supervisor

Sara Balik

Mark Popovsky

Date July 2 19 76

ABSTRACT

Early chick embryos (stages 1 and 4-5; Hamburger and Hamilton, 1951) were used in this study to elucidate ultrastructural and biochemical changes occurring on the cell surface during the development of the primitive streak. Prior to the formation of the primitive streak, the blastoderm consists of two layers, the epiblast and the primary hypoblast (stage 1). During the first 22 hours of incubation, differentiation and morphogenetic movements occur which bring about the formation of the primitive streak, a structure responsible for the formation of the three germ layers. At the time of primitive streak formation, the blastoderm is three-layered: epiblast (presumptive ectoderm), mesoblast (presumptive mesoderm) and hypoblast (presumptive endoderm). However, this hypoblast has received cells invaginated through the primitive streak and hence is now termed the secondary hypoblast. The primary hypoblast has moved peripherally and will become extra-embryonic endoderm.

The cell surface has been implicated in the processes of morphogenetic movement and differentiation as possibly being a control site for cell movement, cell recognition and cell growth. The distribution and biochemical composition of oligosaccharide residues present as glycoproteins have been studied as possible molecular mechanisms for such control. In this context lectins such as Concanavalin (Con A), with the ability to bind specific oligosaccharide residues on the cell surface, have been used to study the distribution of glycoproteins on the cell surface.

Con A, which binds α-D-mannoside and α-D-glucoside residues,

was used in this study to label the cell surface. Cytochemical techniques were then used to render the Con A electron dense and visible by means of electron microscopy. Horseradish peroxidase and diaminobenzidine were used to label cell-bound Con A molecules *in situ* and ferritin coupled covalently to Con A prior to treatment of tissue was also used. These two techniques allowed for appropriate comparisons between protocols to ensure that the patterns observed were not artefacts.

The distribution of Con A on the surfaces of the epiblast and hypoblast was studied in stage 1 and stage 4-5. It was found that all surfaces which were accessible to Con A showed the presence of Con A binding sites (CABS) in stage 1 embryos. The ventral surface of the epiblast displayed a very intense staining reaction, which may reflect the development of a basement lamina on this surface. The distribution of CABS in stage 4-5 was different from that expressed by stage 1 embryos. The dorsal surface of the epiblast displayed more CABS and the ventral surface of the hypoblast displayed virtually no CABS. As well, the ventral surface of the epiblast and the dorsal surface of the hypoblast displayed no CABS, although this may reflect an alteration in the cellular junctions which occur between hypoblast cells, hence rendering these surfaces inaccessible to the lectin probe. The alteration in binding pattern found on the ventral surface of the hypoblast in stage 4-5 embryos reflects biochemical differentiation. The significance of alterations in CABS is discussed with respect to cellular movement and cellular communication during morphogenetic movements and differentiation in development.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Esmond Sanders for the supervision of the work on which this thesis is based. His constructive criticism and guidance throughout the course of this project proved invaluable in the successful completion of it.

I thank my family and friends for enabling me to maintain my sense of perspective during these past eighteen months. They provided encouragement and understanding when I needed it and made the whole undertaking more enjoyable by their very presence.

I would also like to thank the following individuals:

Yvonne Valgren and Sita Prasad for technical advice during the course of this project.

Fred Loeffler and Ken Burt for their help in the printing.

Tella White for her excellent typing skills in preparing the thesis.

TABLE OF CONTENTS

	Page
ABSTRACT	iv
ACKNOWLEDGEMENTS	vi
LIST OF TABLES	ix
LIST OF FIGURES	x
INTRODUCTION	1
BACKGROUND AND LITERATURE SURVEY	4
1. Early Chick Development	4
2. Cell Surface	12
3. Lectins and Concanavalin A	21
4. The Use of Concanavalin A in Developmental Systems	29
a) Effects on Development	29
b) Distribution of CABS and Agglutinability	32
MATERIALS AND METHODS	40
1. Preparation of Embryos	40
2. Electron Microscopy	41
a) Transmission Electron Microscopy	41
b) Scanning Electron Microscopy	44
3. Experimental Procedures	44
a) Concanavalin A-Horseradish Peroxidase-Diaminobenzidine	45
b) Ferritin-Concanavalin A	47
c) Iron Dextran-Concanavalin A	47
d) Fluorescein Isothiocyanate-Concanavalin A	48

Page

RESULTS ---- 49

1. Light and Scanning Electron Microscopy: Description of Areas Sectioned ---- 49

2. Concanavalin A-Horseradish Peroxidase-Diaminobenzidine ---- 58

3. Ferritin-Concanavalin A ---- 92

4. Iron Dextran-Concanavalin A ---- 115

5. Fluorescein Isothiocyanate-Concanavalin A ---- 115

DISCUSSION ---- 118

BIBLIOGRAPHY ---- 132

LIST OF TABLES

Table	Description	Page
1	Summary of results of incubation with Concanavalin A-horseradish peroxidase-diaminobenzidine	60
2	Summary of results of incubation with Ferritin-Concanavalin A	93

LIST OF FIGURES

Figure	Description	Page
1	Schematic representation of tetrameric structure of Concanavalin A	25
2	Scanning electron micrograph of stage 4-5 embryo	42-43
3,4	Scanning electron micrographs of stage 1 embryo showing surface morphology	50-51
5,6	Scanning electron micrographs of stage 4-5 embryo showing surface morphology of primitive streak region	52-53
7,8	Light micrographs of stage 1 embryo	54-55
9,10,11	Light micrographs of stage 4-5 embryo	56-57
12-21	Transmission electron micrographs of stage 1 embryos treated with Concanavalin A-horseradish peroxidase-diaminobenzidine	64-73
22-31	Transmission electron micrographs of stage 4-5 embryos treated with Concanavalin A-horseradish peroxidase-diaminobenzidine	74-83
32-35	Transmission electron micrographs of embryos treated with Concanavalin A-horseradish peroxidase-diaminobenzidine and α-methyl-D-mannoside; control embryos	84-87
36,37	Transmission electron micrographs of stage 1 embryos separated into epiblast fragments and treated with Concanavalin A-horseradish peroxidase-diaminobenzidine	88-89
38,39	Transmission electron micrographs of stage 1 embryos separated into epiblast fragments and treated with Concanavalin A-horseradish peroxidase-diaminobenzidine and α-methyl-D-mannoside; control embryos	90-91
40-49	Transmission electron micrographs of stage 1 embryos treated with ferritin-Concanavalin A	95-104
50-58	Transmission electron micrographs of stage 4-5 embryos treated with ferritin-Concanavalin A	105-112
59,60	Transmission electron micrographs of embryos treated with ferritin-Concanavalin A and α-methyl-D-mannoside; control embryos	113-114
61,62	Transmission electron micrographs of stage 1 embryos treated with iron dextran-Concanavalin A	116-117

INTRODUCTION

The current concept of the plasma membrane is embodied in the fluid mosaic model. Singer and Nicolson (1972) envisaged a fluid lipid bilayer in which globular integral proteins are embedded, either spanning one or both leaflets of the bilayer. A second group of proteins, peripheral proteins, is attached to the membrane by weak non-covalent bonds. The globular protein molecules as well as the lipids are amphipathic, that is, they have a hydrophilic (polar) and a hydrophobic (non-polar) end. This allows for an asymmetry in the membrane as a result of their interactions in two phases, i.e., aqueous and hydrocarbon. A third class of membrane components, oligosaccharides, is present mainly as side chains attached covalently to protein or lipid. These side chains are hydrophilic and are generally found on the non-cytoplasmic side of the membrane (for example, Nicolson and Singer, 1974). The principal monosaccharides of these side chains include galactose, mannose, fucose, galactosamine, glucosamine, glucose and sialic acid (for a review of glycoproteins in membranes see Hughes, 1973). Although these side chains are usually no more than fifteen sugar residues long, they are branched and bonded through a variety of linkages which can give rise to potentially great numbers of structures. As such, these carbohydrate side chains linked to either protein or lipid are attractive candidates for endowing specificity to cell surfaces.

Recent studies (for a review see Nicolson, 1976) indicated that these glycoproteins are mobile in the plane of the membrane. The relative mobility of these groups could be modulated by any combination

of the following: protein or lipid associations in the plane of the membrane, sequestration or exclusion of components in or from specific lipid regions, association either at the inner or outer surface with peripheral proteins, or control through membrane-associated cytoskeletal components. As well, the fluidity of the lipid bilayer may also be altered, for example, by the addition of cholesterol which could affect lipid phase transitions (Nicolson, 1976). Any of these mechanisms could affect the mobility of these glycoproteins in the fluid-lipid matrix and thereby change the topographical distribution of surface groups.

The possibility of regulatory mechanisms existing to control the distribution of oligosaccharide residues relates directly to the possible control of cell movement, cell recognition and cell growth, and hence to problems in morphogenesis and differentiation in developmental systems. For example, the structural organization and/or the topographical distribution of these oligosaccharide side chains could directly affect specific adhesion between cells or cell groups, or may aid in cell recognition or communication between groups. Alterations in either structure or distribution of a subpopulation of embryonic cells could conceivably change the manner in which these subpopulations relate to each other. Whether or not these oligosaccharide groups are directly related to the process of morphogenesis, it is possible that they might serve to allow discrimination between cell populations hitherto considered as undifferentiated.

Therefore, lectins such as Concanavalin A (Con A) which bind to specific cell surface groups can be used as probes with which to

study changes in distributions of these receptor sites. It was the purpose of this study to elucidate the distribution of Con A binding sites (CABS) on the cell surface of the early chick embryo. The relative amounts of receptors could be compared on surfaces of corresponding cells in unincubated and gastrulating embryos by the use of ultrastructural cytochemical techniques. A change in the binding of Con A on any surface would indicate alterations in the complement of cell surface receptor sites, which could in turn relate to the morphogenetic events of that particular cell group or to the state of differentiation of subpopulations of the embryo.

BACKGROUND AND LITERATURE SURVEY

1. *Early Chick Development*

The chick embryo undergoes cleavage as it passes down the hen's oviduct. At the time of laying, the germ is a disc with two areas generally discernible, the center thinner area pellucida, surrounded by the thicker area opaca. The area pellucida, in a freshly laid egg, is essentially a single layer of cells (Eyal-Giladi and Kochav, 1976), although there is wide variability as hens retain their eggs in their oviducts for greater or lesser periods of time. Bellairs (1971) suggests that external factors such as length of daylight at different times of the year or even a traumatic experience may affect the hormonal control of the laying cycle. Nevertheless, Eyal-Giladi and Kochav (1976) have devised a manual extraction technique yielding germs still undergoing cleavage, which they claim circumvents these problems. It was found that eggs could be removed from the oviduct by pressure applied to the abdomen approximately 5.5 hours after the previous egg was laid. However, by the time most investigators receive their fertilized eggs, the area pellucida has become two-layered (Fig. 8). This is due to slow development of unincubated eggs left at room temperature. The germ in the region of the area pellucida has become two-layered, the superficial epiblast, a layer of columnar epithelia-like cells, and the underlying hypoblast, which is not yet a coherent layer except in posterior regions of the embryo. As well, in the posterior region of the area pellucida, a small opaque crescent, the embryonic shield, is found. This is an area

in which the cells are as much as five deep (Bellairs, 1971). By this time the embryo has attained bilateral symmetry, as judged by the appearance of the area pellucida (Eyal-Giladi and Kochav, 1976). This is stage 1 according to Hamburger and Hamilton (1951), but others have subdivided Hamburger and Hamilton's stages 1 and 2 into as many as fourteen stages (Eyal-Giladi and Kochav, 1976), encompassing three distinct developmental periods: cleavage, formation of the area pellucida (period of symmetrization), and period of hypoblast formation. It should be understood that the first two periods have largely been ignored in the literature due partly to the problems of collecting these early stages and partly due to a lack of understanding of the importance of these early stages in development. Most of the experimental studies have involved chick blastoderms already at the primitive streak stage and older, based on the tacit understanding that it was at these stages that differentiation and morphogenesis started. Recent work has indicated the need to take a closer look at earlier stages as it is becoming increasingly evident that differentiation occurs in what was previously considered to be undifferentiated stages (Bellairs, 1971; Bancroft and Bellairs, 1974; Jacob *et al.*, 1974; Eyal-Giladi and Kochav, 1976).

The mechanism of formation of a coherent layer of hypoblast is a controversial point in the literature. To add to the confusion, it is now generally agreed that there are at least two contributions to the hypoblast which form primary and secondary hypoblast respectively during development (Rosenquist, 1966; Vakaet, 1970; Bellairs, 1971). It is the formation of the primary hypoblast which is most controversial

since most workers agree that the addition of cells from the primitive streak to the secondary hypoblast is by invagination of epiblast cells through the streak.

Peter (1938), as cited by Bellairs (1971), initiated the theory of delamination which proposed that the hypoblast resulted from a separation of a continuous sheet of cells from the lower surface of the epiblast. This separation is supposed to start in the posterior region of the embryo and progress anteriorly. A similar theory, polyinvagination, supported the idea that individual cells migrated ventrally and subsequently joined to form a coherent layer (Merbach, 1935, as cited by Eyal-Giladi and Kochav, 1976; Vakaet, 1962; Spratt and Haas, 1965; Eyal-Giladi and Kochav, 1976). Merbach (1935) proposed an additional mechanism, that of an invagination of cells at the posterior end of the embryo. Vakaet (1962) and Spratt and Haas (1965) suggested that in addition to polyinvagination, there was an anteriorly-directed movement of hypoblast cells from Koller's sickle, a posterior concentration of cells. These authors agree on the existence of Koller's sickle, but do not agree that a furrow is present, homologous to the amphibian blastopore, through which invagination occurs, as originally suggested by Koller (1882, as cited by Eyal-Giladi and Kochav, 1976). Others have also proposed the existence of a "blastopore" in some region of the blastoderm (for example Duval, 1884, as cited by Eyal-Giladi and Kochav, 1976; Jacobson, 1938).

Opposed to these two theories (polyinvagination and delamination) of the hypoblast originating from the lower surface of

the area pellucida is the idea of an origin from the germ wall (area opaca). Disse (1878, as cited by Eyal-Giladi and Kochav, 1976) postulated the idea of a gradual formation of hypoblast from the periphery towards the center in a concentric fashion.

Eyal-Giladi and Kochav (1976) described the formation of the primary hypoblast as follows: At a time when the area pellucida is completed, the primary hypoblast is starting to form as isolated clusters of cells (Fig. 7), more crowded at the posterior end of the germ. Separating these clusters from the anterior area opaca is a transparent belt-like pure epiblast region, and later an increase in cell density at the posterior forms a clearly demarcated sickle (Koller's sickle). Anteriorly, the non-coherent cell clusters continue to grow in size and with more time the sickle becomes coherent with aggregates of the posterior half forming in the posterior region of the embryo, the embryonic shield. The sickle and transparent belt behind it are well defined, and the hypoblast, although sheet-like, is not continuous and gives the impression of being formed by the fusion of cell masses. Eyal-Giladi and Kochav (1976) believe the primary hypoblast results from an orderly coalescence of cell aggregates which have polyinvaginated. They suggest there may also be an active anterior movement, although they have no data for this. This is in agreement with others (Vakaet, 1962; Spratt and Haas, 1965). Eyal-Giladi and Kochav (1976) also described the development of a cellular bridge which appears and connects the central region of the posterior sickle with the posterior area opaca. It is within this cellular bridge that the rudiment of the primitive streak appears.

In a scanning electron microscope study, Bancroft and Bellairs (1974) reported the appearance of "crypts" in unincubated embryos. These "crypts" separate adjoining cells and have microvilli from the cells protruding into their interior. They are found randomly over the blastoderm, but disappear by Hamburger and Hamilton stage 2. Bancroft and Bellairs (1974) believe each "crypt" may be the site of an invagination.

The problem of how the germ layers form within the area pellucida and the cell movements which occur and lead to gastrulation has been studied through fate maps. A variety of techniques, including the use of vital dyes, particulate matter, time-lapse cinematography and grafting of radioactively-labelled tissue squares to homologous sites in recipient embryos, have been used to construct these fate maps (for example Peter, 1938, as cited by Bellairs, 1971; Spratt and Haas, 1960; Rosenquist, 1966; Vakaet, 1970). There is general agreement that morphogenetic movements occur within a few hours of incubation and that there is a movement of cells towards the posterior median end of the blastoderm (Bellairs, 1971). It is believed by some (Eyal-Giladi and Wolk, 1970; Eyal-Giladi, 1970; Eyal-Giladi *et al.*, 1975a) that the hypoblast induces the formation of the primitive streak.

Fate maps have led to the following description of the formation of the primitive streak: A convergence of epiblast cells towards the midline in the posterior half of the area pellucida initiates the formation of the primitive streak. As this concentration of cells proceeds, the parts of the epiblast situated farther out

laterally and anterolaterally swing in a curve backwards and inwards, taking the place of epiblast cells which have shifted to the midline. The short primitive streak elongates by the concentration of more and more cells to the midline and contracts from a broad, vaguely delineated structure to a narrower structure called the definitive primitive streak. During this process the primitive streak further elongates. Cells of the epiblast as early as the short primitive streak stage have started to invaginate between the epiblast and hypoblast. There is no infolding as found in amphibians, but rather a downward movement of individual cells. As the migrating cells reach the hypoblast, they intermix with it (Fig. 11). At this point the entire primitive streak is a mass of movement, cells migrating firstly downward from the surface towards the hypoblast and secondly, anterolaterally between the epiblast and hypoblast.

Along the middle of the fully developed primitive streak runs a narrow furrow, the primitive groove, and at the anterior end of the primitive streak is a thickening, Hensen's node (Fig. 2). The center of Hensen's node is excavated to form a funnel-shaped depression. These structures are due to the mass movement of cells from the surface of the blastoderm into the interior. Although single cells migrate downward through the streak, sheets of epiblast cells move towards the primitive streak in a coordinated fashion, with whole areas of the epiblast moving towards the midline and into the primitive streak to be replaced in turn by others.

Rosenquist (1966) demonstrated the migration of epiblast to the streak and movement ventrally of these cells to penetrate into the

hypoblast and form the secondary hypoblast. At stage 3 (Hamburger and Hamilton, 1951) all cells which invaginate enter the hypoblast and are presumptive endoderm. As development proceeds (stage 4), increasingly larger amounts of presumptive mesoderm gain access to the streak. The secondary hypoblast pushes forward and outward, and this results in an extensive area of hypoblast around and in front of Hensen's node originating from cells derived from anterior regions of the primitive streak. This region later shifts forward to form the foregut (Rosenquist, 1966, 1971). Endoderm laying more posteriorly in the primitive streak after invagination moves more laterally and even laterocaudally to replace the primary hypoblast. This gives rise to the lining of the yolk sac (Rosenquist, 1966). The presumptive notochord cells are concentrated in the deeper parts of Hensen's node after invagination at the cephalic end of the streak and then move in the midline anteriorly (Rosenquist, 1966).

Bancroft and Bellairs (1974) reported the appearance of "dimples" in scanning electron micrographs. These "dimples" were found in the posterior end of the area pellucida of stage 5 embryos. They were localized depressions found at the junction of several cells, which were arranged in a rosette-like array surrounding it. Bancroft and Bellairs interpreted these "dimples" as sites of invagination.

The primitive streak, while active, is a mass of cells continuous with both the epiblast and the hypoblast, with mesoderm cells migrating between the two (Fig. 11). It should also be noted that the primary hypoblast has been altered by the immigration of endodermal cells from the epiblast. These newly immigrated cells contribute to

the gut and its derivatives.

Morphological evidence based on scanning electron microscopy has given further support to the idea that differentiation has occurred in what was previously thought to be undifferentiated cells. Bancroft and Bellairs (1974) report that regional differences occur in the blastoderm as early as stage 2 (Hamburger and Hamilton). They find that the central region possesses fewer microvilli but more vesicles than cells in the posterior end. Vakaet (1970) had previously noted that when living embryos were viewed by transmitted light, this central area was relatively thicker and denser than peripheral regions. In the stage 5 embryo, Bancroft and Bellairs (1974) found that the regions poor in microvilli but rich in globular projections and vesicles corresponded to areas which would invaginate to form neural plate, some endoderm, somites and lateral plate mesoderm. The areas which were found to be rich in microvilli would become ectoderm and extra-embryonic tissues. From a correlation between fate maps and maps showing these areas, Bancroft and Bellairs concluded that cells which would later invaginate to form internal organs begin to differentiate from future ectodermal cells almost immediately upon incubation.

These authors and Jacob *et al.* (1974) also describe the appearance of threads. These threads vary in length between 3 and 17 µm and may or may not have "beads" measuring up to 0.75 µm in diameter. Bancroft and Bellairs (1974) suggest several possible functions. The threads may provide a means of communication between cells which are not immediate neighbours. This could have some significance in coordinating developmental activity. By allowing a

cell to "communicate" with cells which are more distant neighbours, a better assessment of the cells' relative developmental progress could be made. An alternate function could be to serve as an aid to migration of mesoderm cells, allowing the cell to explore for suitable pathways on which to move.

Additionally, vesicles were seen in transmission electron micrographs at the junction of two cells (Figs. 56, 57) (Balinsky and Walther, 1961; Bancroft and Bellairs, 1974). Bancroft and Bellairs believe that these intracellular vesicles are formed when processes from two adjacent cells meet, enclosing a droplet of fluid. During the first two days of incubation, these vesicles develop in size and number. Ruggeri (1966, as cited by Bancroft and Bellairs, 1974) suggested that these vesicles may be involved in absorption of albumen.

2. *Cell Surface*

The cell surface plays an important role in the control of cell growth, cell movement and cell recognition and is consequently intimately involved with differentiation and morphogenesis. Initial interactions between a cell and its immediate environment will occur at the cell surface, and these "messages" received by the cell surface could be transmitted to the cytoplasm, perhaps altering cellular activities such as synthesis. A change in synthesis may in turn be reflected in alterations of the cell surface. Such sequences as this might be the driving force behind differentiation and development in embryonic systems.

There has been some work in recent years which has directed

its attention to alterations in cell surface properties of chick cells at early stages of development. Zalik *et al.* (1972) reported differences in surface charge density between prestreak cells and primitive streak cells. They reported a progressive decrease in electrophoretic mobility as the primitive streak developed. This decrease in surface charge density occurs at the time when active cell migration occurs. The surface charges which account for electrophoretic mobility arise from ionogenic groups such as carboxyl, amino and phosphate groups (Finean, Coleman and Michell, 1974).

Lanthanum nitrate and colloidal iron have been used to demonstrate the presence of surface material on chick embryos using electron microscopy (Sanders and Zalik, 1972). These staining methods do not have precise specificities but it is believed that they stain protein-polysaccharide material (for a review see Martínez-Palomo, 1970). Sanders and Zalik (1972) reported that surface staining with lanthanum nitrate of whole embryos of stage 1 generally showed a heavier staining than embryos of stage 5. They also mentioned that the cells found at the base of the primitive groove of stage 5 embryos show a markedly greater staining intensity than the rest of the embryo. No staining was found on mesoderm cells in the primitive streak region. Sanders and Zalik (1972) speculated that the presumptive mesoderm cells may rapidly synthesize new surface material upon reaching the primitive streak region and upon the completion of migration this material is then lost. The distribution of lanthanum nitrate was not uniform on whole embryos, which Sanders and Zalik (1972) believe reflects an inhomogeneity of surface material. This uneven distribution

is in contrast to the even dense layer of stain found on EDTA-dissociated blastoderms of stages 1 to 5. The colloidal iron staining technique was reported to demonstrate a similar loss in affinity for surface binding with increasing age of cells. This technique did not stain the surface of dissociated cells evenly; instead localized patches of dense staining were found. The differences in distribution of lanthanum nitrate staining on whole embryos and dissociated cells was thought to represent a redistribution of the cell surface material which occurred during the dissociation procedure (Sanders and Zalik, 1972).

More recent work has dealt with the aggregation and sorting out of dissociated chick cells taken from early developmental stages. This aggregation and sorting out is believed to result from surface properties which allow cells to selectively adhere and thus distinguish between similar and dissimilar cells. This technique is well established (for a review see Weiss, 1967), and it is generally assumed that cell aggregation *in vitro* and the resulting sorting out is a valid experimental method for studying cell recognition, selective cell adhesion and cellular interactions (Moscona, 1974).

It was generally agreed that differentiation initially starts with the onset of gastrulation and thus initial work was done with definitive streak stages of the chick (for example see Zwilling, 1963). From the work described by Sanders and Zalik (1972) and the information that the first morphogenetic movements in the chick were the formation of the primary hypoblast (Eyal-Giladi, 1970), it was felt that cells from prestreak blastoderms would possess surface

characteristics which would allow them to sort out into two distinct groups and continue to differentiate (Zalik and Sanders, 1974; Eyal-Giladi *et al.*, 1975b; Macarak, 1975; Sanders and Zalik, 1976).

Zalik and Sanders (1974) reported that aggregates prepared from cells dissociated by EDTA or trypsin from whole non-incubated blastoderms segregated to form two cell populations. The aggregates were initially solid, but with time formed cavities which were bordered by loosely-packed cells forming a continuous phase. Within this continuous phase were groups of more tightly-packed cells which constituted the second cell type. On the basis of morphological similarities and the observation that the loosely-packed phase differentiated with time into cells resembling yolk sac endoderm, Zalik and Sanders (1974) suggested that these cells originated from the hypoblast. The compact phase was thought to originate from the epiblast, based upon the formation of junctional complexes found which are similar to those found in the epiblast *in situ*. Their conclusion was that two populations of cells existed in the unincubated chick blastoderm, each with distinct surface characteristics (Sanders and Zalik, 1976).

In a similar study, Eyal-Giladi and co-workers (1975b) provided additional evidence for the existence of two distinct cell populations in the unincubated chick blastoderm, even though there were differences in technique. These investigators used very precise staging techniques in which only blastoderms with a complete primary hypoblast were used (staging in accordance with Eyal-Giladi and Kochav, 1976). As well, all reaggregation experiments were carried out with

either the hypoblast or epiblast radioactively labelled. The aggregates of Zalik and Sanders (1974) attained a rounded shape, which was probably due to culture in suspension in a fluid medium in agitated flasks. Eyal-Giladi and her co-workers cultured their aggregates on a vitelline membrane over a solid culture medium, which enabled their aggregates to form a flattened shape. They used this technique on the assumption that the behaviour of cells would approximate more closely the normal behaviour. Despite these differences in technique, two layers of cells sorted out: the uppermost layer composed of epiblast and the lowermost layer composed of hypoblast cells. This was opposite to the expected arrangement in that it was thought that the epiblast would attempt to establish contact with the vitelline membrane. These investigators suggested that the hypoblast may tend to migrate away from the gaseous phase, or alternately these cells are attracted to the source of nourishment. The evidence from all this work seems to be conclusive in that cells from the early blastoderm are sufficiently differentiated or possess surface characteristics which allow them to sort into two cell populations (Zalik and Sanders, 1974; Eyal-Giladi *et al.*, 1975b; Sanders and Zalik, 1976).

The molecular basis for the expression of these cell surface differences appears to lie in surface receptors or recognition sites which may be of a carbohydrate nature (for review see Winzler, 1970; Hughes, 1973). Various theories have been postulated, which suggest that these specific components on the cell surface function as receptor sites for ligands which mediate cell recognition and selective cell adhesion. Both of these phenomena could be involved in morphogenetic

movement.

Early workers (Tyler, 1946; Weiss, 1947, as cited by Moscona, 1974) had independently suggested that the cell surface might contain complementary molecules which could interact specifically in a lock and key fashion. Weiss (1947, as cited by Moscona, 1974) postulated two functions for these molecules. They could function in intercellular recognition, detecting likeness if both members of each matching pair were bound to a particular cell type. This would allow a cell to "test" its immediate environment and then respond in an appropriate manner. A second function of these complementary molecules would be in mediation of cell communication if the release of soluble molecules by the cell could bind to its complementary receptors on other cells.

In more recent studies, Moscona has suggested a very similar hypothesis: the cell-ligand hypothesis (Moscona, 1974). Cell-ligands are cell-linking components at the cell surface, assumed by Moscona to be glycoprotein in nature. Cells with a high degree of ligand complementarity would show positive recognition through the linking of ligands, whereas cells with little ligand complementarity would exhibit negative recognition. The hypothesis stated that the characteristics of the cell ligands would evolve during differentiation and the alterations in ligand patterns account for differential adhesiveness and selective cellular affinities.

Moscona and co-workers have isolated cell aggregating factors (for review see Moscona, 1974) which are tissue specific in enhancing reaggregation and promoting the further differentiation. It was found that in the case of retina factor obtained from embryonic

retina cells that the fully differentiated retina cells did not release the retina factor. These fully differentiated retina cells also would not respond to the aggregating factor obtained from younger cells. It appeared from this evidence that upon completion of differentiation and morphogenesis, these cells are no longer capable of reforming the surface components which were required for morphogenesis at earlier stages of development (Moscona, 1974).

The mechanism of binding of ligand to the cell surface has been under study. Moscona (1968) had suggested that the cell ligands bind to acceptor sites on the cell membrane. Weinbaum and Burger (1973) have also provided evidence for a two-component system in the case of sponge cell aggregation.

While the detailed chemistry and structure of these cell ligands is unknown, there is some evidence which indicates that they are glycoprotein in nature. They contain relatively high amounts of glutamic and aspartic acid residues, as well as mannose, galactose, glucosamine and sialic acid moieties (Moscona, 1974). However, the differences which account for the specificities, mechanism of linking cells, their biosynthesis and distribution on the cell surface is largely unknown. As well, the nature of the acceptor sites and the mechanism of the two-component ligand-acceptor complex is also largely unknown. However, recent work (McDonough and Lilien, 1975b) has suggested that aggregation-promoting material which is specific for the retina and cerebral lobe (RAPM and CLAPM) bind to the respective tissue by sugar residues. Specifically, the binding of RAPM to retina cells was dependent on a terminal N-acetyl-galactosamine residue and

binding of CLAPM to cerebral lobe cells was dependent on a terminal mannosamine residue.

If the nature of the ligand is glycoprotein, then the mechanism of interaction which would link cells could be either protein-protein, protein-carbohydrate or carbohydrate-carbohydrate interactions. The presence of glycosyltransferases and their acceptors on the cell surface has been suggested by some (for example Roseman, 1970; Roth *et al.*, 1971; for a recent review see Shur and Roth, 1975) to be a possible molecular basis for these interactions.

Glycosyltransferases are enzymes which catalyze the transfer of monosaccharide residues from a sugar donor (sugar nucleotide) to the non-reducing terminus of a specific sugar acceptor. They are named according to the sugar donors they utilize (Shur and Roth, 1975). Although no transferase can utilize more than one sugar donor, the acceptor specificities are less stringent. A wide range of possible functions for these enzymes has been suggested, including termination of carbohydrate portion of ligands, stabilization of membrane components by cross-linking, transport of sugars across the cell membrane, degradation and turnover of cell ligands as well as the binding of ligands to acceptors. There has been evidence for the presence of transferases on the cell surface in a number of developmental systems, including gastrulating chick embryos (Shur and Roth, 1973). Using ^{3}H-labelled sugar nucleotides and autoradiographs of serially sectioned chick embryos (stage 9+-14) it was found that the migrating and inductive cell types expressed the most active surface transferase activities (Shur and Roth, 1973, 1975). It was concluded that sugar

nucleotides were the substrates and that the final glycosylated products of the various transferases were externally located at the cell surface. The most active glycosyltransferases were galactosyl-, N-acetyl glucosamine- and fucosyl-transferase. The age of the embryos assayed was relatively late, but these authors concluded that the temporal and spatial patterns of specific activity of glycosyl-transferase was suggestive of the localization of these enzymes on cells undergoing migratory movements or inductive interactions (Shur and Roth, 1975).

Studies involving glycosyltransferases are often criticized for the lack of proper controls. Controls must be done to ensure that hydrolysis of sugar nucleotide has not occurred extracellularly, and the breakdown product internalized for synthesis. As well, it must be determined that labelled sugar nucleotides are not simply binding to the cell surface. It has been suggested that excess unlabelled sugars and sugar phosphates, assays for the rate of free labelled sugar incorporation, use of metabolic poisons and tests for the hydrolysis of sugar nucleotides are required before the identity of the sugar donor as sugar nucleotide can be established (Shur and Roth, 1975). If the presence of these enzyme systems is confirmed at the cell surface, then the glycosyltransferase model may still not be the molecular basis for the cell-ligand hypothesis. Alternative interpretations of the data could be the following: the transferases may not be directly involved in controlling cellular interactions but rather may function to synthesize other surface molecules which would be directly involved. It is also possible that other types of

enzyme-substrate pairs could mediate most of the cellular interactions, for example glycosidases, proteinases or kinases (Roth, 1973).

Taken together, the evidence seems to indicate the involvement of cell surface components in morphogenesis and differentiation. The problem exists whether the subtle differences in cell surfaces can be solely explained by simple chemical differences between ligands. It has been suggested that the required diversity of cell surface specificities could be met if temporal-topographical distribution patterns were added to the dimension of chemical differences. Thus, the biochemical composition of the ligand plus the distribution of it over the cell surface at any point in time may both be important in the involvement of the cell surface during development (Moscona, 1974).

3. *Lectins and Concanavalin A*

In recent years, lectins have been used as molecular probes for sugar residues on the cell surface. Lectins are a diverse group of protein molecules isolated from a wide variety of plants as well as some invertebrates, fish and micro-organisms. They share in common one feature: binding sites for specific sugar residues, to which they bind tightly and reversibly. This binding can be inhibited by the addition of the specific sugar to the assay. The specificity of the sugar as a hapten inhibitor is taken as evidence for the presence of sugar receptors for the lectin on the cell surface with a similar but not identical structure to the hapten inhibitor. These receptors are either glycoprotein and/or glycolipid; both are important constituents of the cell membrane (Bretscher and Raff, 1975).

Lectins have been found to have a wide variety of effects on cells, including cell agglutination, induction of mitogenesis and toxicity. Due to their sugar-binding specifities, they have also been used for studies on blood group substances, analysis of surface structure of normal and transformed cells, specific isolation of glycoproteins or other sugar-containing molecules and in antigen-antibody models (see review see Lis and Sharon, 1973; Nicolson, 1974). As well, various techniques have been developed which label the lectin to facilitate the microscopical detection of the lectin bound to the cell surface. The visualization of a lectin permits the study of not only the presence but also the distribution of the lectin receptors on the cell surface.

It has been accepted that the nature of the saccharide-binding specificities could be adequately described by inhibition studies utilizing either simple sugars or glycosides as hapten inhibitors. However, the best inhibitor is not necessarily identical or similar to the cell surface lectin receptor, nor does the lectin necessarily bind only to terminal non-reducing sugar residues. These inhibitors may only mimic a carbohydrate which occurs in the lectin receptor on the cell surface. It is now known that cell surface oligosaccharides are complex structures which may be influenced by neighbouring oligosaccharides through non-covalent interactions such as hydrogen bonds, the nature of the linkages of the saccharides in the oligosaccharide chain and the properties of the protein backbone to which the chain is linked (Lis and Sharon, 1973; Nicolson, 1974). Evidence has been presented which indicates that some lectins can interact with terminal

residues as well as with internal core sugars (for example Goldstein *et al.*, 1973). Although no studies on glycolipids as lectin receptors have been done, it is probable that these may also function as lectin receptors. Work is currently in progress in several laboratories to isolate cell surface lectin receptors and it would be expected that these isolated receptors would be potent inhibitors of lectin binding (Sharon and Lis, 1975).

One of the most widely studied lectins is Concanavalin A (Con A), which has been isolated from the jack bean, *Canavalia ensiformis*. The biological activities of Con A can be inhibited by the addition of α-methyl-D-mannoside. Goldstein and his co-workers (1973) have reported that Con A binds specifically to non-reducing terminal α-D-mannopyranosyl, α-D-glycopyranosyl and α-D-fructofuranosyl residues as well as non-terminal α-D-mannopyranosyl residues (as well as their glycosides and other sterically related structures).

Con A is a protein composed of identical subunits, each with a molecular weight of 25,500. The association of these subunits is pH-dependent with dimers forming predominantly below pH 6.0 and tetramers above pH 7.0 (Reeke *et al.*, 1975). At higher pH, the formation of tetramers is accompanied by the time-dependent development of turbidity which indicates the formation of higher aggregates (McKenzie, Sawyer and Nichol, 1972). This can be eliminated at pH 7 by either an increase in ionic strength or by the addition of glucose (McKenzie, Sawyer and Nichol, 1972).

Low resolution crystallographic studies have indicated that the protomers are "gum-drop" shaped with the base measuring approximately

40x39 Å and with a height of 42 Å (Reeke *et al.*, 1975). The Con A subunit is globular, composed of 238 amino acids, with four of these units interacting to form a tetrahedral shape. The predominant structural feature of each subunit is an extended polypeptide chain arranged in two antiparallel sheets (β structures). One of these sheets interacts with other subunits to stabilize dimer and tetramer structures (Lis and Sharon, 1973; Nicolson, 1974; Reeke *et al.*, 1975). Residues which are not included in the β structure are in regions of random coil.

Each subunit binds one calcium and one manganese ion plus one saccharide molecule. The metal ions are necessary for saccharide binding activity. It has been shown that treatment of Con A with ethylenediamine tetracetic acid (EDTA) destroys saccharide binding activities which can be restored by addition of $MnCl_2$ and $CaCl_2$ (Yariv *et al.*, 1968; Uchida and Matsumoto, 1972). These binding sites have been proposed to be located in the subunit as indicated in Figure 1. It was believed that the saccharide binding site is located in a pocket located on each subunit and surrounded by hydrophobic amino acid residues, but recent evidence raises some doubt as to whether these cavities are the actual saccharide-specific binding sites. Becker *et al.* (1976) have proposed that a second site located near the calcium and manganese binding sites is responsible for binding specific saccharide residues. There is some controversy in the literature indicating that binding sites observed in the crystal may not be the same as the inhibitory sugar-binding site in solution (for review see Reeke *et al.*, 1975; Becker *et al.*, 1976).

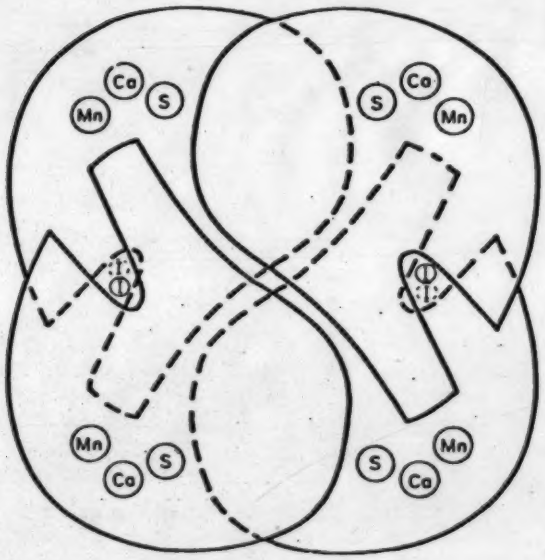


Fig. 1. Schematic representation of tetrameric structure of Con A. The proposed binding sites for manganese and calcium are indicated by Mn and Ca, the saccharide binding site near the metals is indicated by S and the hydrophobic binding sites in the cavity by I. (From Becker *et al.*, 1976).

Recent evidence (Huet *et al.*, 1975) has suggested that the Con A molecule undergoes quaternary structure transitions at different temperatures. At 37°C, the tetrameric form is predominant but at lower temperatures it dissociates to form dimers. It was found in this study that the dimer did not induce agglutination at low temperatures but that it did bind free exogenous glycoprotein. This study suggested that caution should be used when determining the effects of temperature on cell surface receptor sites and agglutination, as the interpretation cannot be solely based in terms of cell surface properties. The steric

effects of dimer-tetramer transitions of the Con A molecule should also be taken into account.

Con A has been used to label cell surfaces in a specific manner. A variety of suitable markers have been developed including various radioactive labels and other compounds. The binding of radioactive labelled Con A can be estimated by scintillation counting or its distribution followed by radioautography. Several other procedures have been developed which allow the visualization of reaction product by light or electron microscopy. These latter labels enable the pattern of Con A binding to be observed on the cell surface (for review see Sharon and Lis, 1975). The use of techniques designed to render the Con A binding sites (CABS) electron dense are especially powerful due to the much greater resolving power of the electron microscope. Thus, it may be determined whether the CABS are randomly distributed, aggregated into clusters, or capped at one pole of the cell. Such variations in distribution may account for a variety of cell surface-mediated effects.

There are two ways in which a label may be attached to the lectin. A label may be covalently attached to the lectin prior to reaction with the cell, or the marker's sugar residues may be attached to the cell-bound lectin. Fluorescein, tetramethyl rhodamine, ferritin and microperoxidase have been covalently bound to Con A. Examples of the second type of labelling procedure include fluorescent antibodies, peroxidase and hemocyanin (for reviews see Sharon and Lis, 1975).

One of the first labelling methods employed peroxidase

(Bernard and Avrameas, 1971). In this method, Con A is allowed to bind to sugar residues on the cell surface, but because these sugar residues are in an insoluble form, the remaining binding sites on Con A remain unoccupied. Horseradish peroxidase (HRP), a glycoprotein, then interacts with the remaining one or more binding sites on the Con A molecule via its sugar residues. The catalytic activity of the HRP is revealed by the method of Graham and Karnovsky (1966). The cell-lectin-HRP complex is treated with hydrogen peroxide and 3,3'-diaminobenzidine tetrahydrochloride. The oxidation of the diaminobenzidine leads to a deposition of an electron opaque reaction product after osmium tetroxide. It is believed that there is some reduction of the osmium tetroxide by the reaction product which renders the reaction product even more electron dense (Sharon and Lis, 1975). This method is sensitive because the enzymatic activity has an amplifying effect, that is, a few molecules of HRP at a site can generate a much larger amount of reaction product.

As in all cytochemical techniques, it is necessary to ascertain that the observed lectin binding is specific for certain sugar residues. It is therefore necessary to carry out each series of experiments with suitable controls. In the HRP technique, an excess of α-methyl-D-mannoside is added to compete with the enzyme and the sugar present on the cell surface for the binding sites of Con A.

A technique has been described in which ferritin is covalently bound to the Con A (Nicolson and Singer, 1971). Ferritin-conjugated Con A was prepared by the glutaraldehyde coupling method of Avrameas (1969). In the present work, Con A was purchased already conjugated to

ferritin, but the conjugation procedure is as follows. The conjugation was carried out prior to incubation with the cell and does not involve any specific interactions between the ferritin and Con A. Con A and ferritin were incubated together in the presence of glutaraldehyde which stabilized any covalent bonds formed. More recent studies (Nicolson and Singer, 1974) have added the specific sugar inhibitor at this point to protect the active sites of Con A. The mixture was then extensively dialysed to remove the sugar inhibitor and large aggregates are separated by centrifugation. Separation of ferritin-Con A (ferr-Con A) from unconjugated proteins was achieved by column chromatography and the ferr-Con A was then concentrated. All of these procedures were carried out in sodium phosphate buffer with the pH lowered to 6.8 to prevent aggregations of Con A forming. The ferr-Con A complex was visualized directly in the electron microscope and seen as discrete particles. Spatial resolution of this technique was limited by the dimension of ferr-Con A complex (approximately 150 Å), and the specificity of the Con A (Nicolson and Singer, 1974). The technique provided a more stoichiometric relationship between the number of reactive sites and the amount of ferritin bound than the HRP technique.

A third technique has been described which uses iron dextran to label Con A (Martin and Spicer, 1974). It is a simple two-step procedure in which incubation in iron dextran follows Con A binding to the cell surface. As the iron dextran complex contains an electron dense core with carbohydrate residues which can bind to Con A, no further treatment is required to visualize the reaction product in the

electron microscope. It was, however, necessary to use relatively high magnifications to view these relatively small particles.

All of the above techniques are used in conjunction with electron microscope. It should be noted that these are only a few of a number of techniques which can be found in the literature (for a review see Sharon and Lis, 1975) for use with transmission electron microscope. Hemocyanin has been used in conjunction with shadow cast replicas to view entire upper surfaces of cell membranes (Smith and Revel, 1972). It has also been reported that the hemocyanin molecule is large enough to be viewed with a scanning electron microscope (Weller, 1974; Brown and Revel, 1976).

As well as electron microscopy studies, Con A has been labelled with fluorescein. This allows visualization with the ultraviolet microscope without any further treatment. Con A is conjugated to fluorescein isothiocyanate (FITC) by the method of Smith and Hollers (1970), in which Con A and FITC are allowed to react and free FITC is removed by passage through a Sephadex column. In the present work the FITC-Con A was obtained commercially.

4. *The Use of Con A in Developmental Systems*

a) *Effects on Development*

Con A has been used in a wide variety of developmental studies. Developmental changes have been reported to occur if the lectin is allowed to interact with living cells during development. Generally, Con A had deleterious effects on development, either slowing development or causing abnormal development and death. Vacquier and

O'Dell (1975) reported that Con A inhibited the dispersion of cortical granule contents in sand dollar eggs following fertilization. During normal fertilization, these granules fused with the egg membrane, extruding their contents into the perivitelline space. The resulting cortical granule material plus the vitelline layer formed the fertilization membrane. If fertilization occurred in the presence of Con A, the vitelline layer elevated and the cortical granules fused with it, expelling the dense cortical material into the perivitelline space, but instead of dispersing it, this dense material remained attached to the elevated vitelline layer. Therefore, this layer remained thin and weak. The eggs do not develop normally, with only a low percentage cleaving.

Lallier (1972) studied the effects of high doses of Con A (0.5-1 mg/ml) on fertilization and cleavage in the sea urchin embryo. He reported that fertilization was inhibited or the formation of the fertilization membrane was disturbed. O'Dell (1972) reported that low doses of Con A (10 µg/ml) affected not only the formation of the fertilization membrane, but also restricted cleavage to nuclear division so that syncytia were produced.

The effects of Con A on vertebrate development have also been studied, for example in the amphibian (*Ambystoma maculatum*) (Moran, 1974a). At high concentrations (0.5-1 mg/ml), development ceased and death resulted, but at lower concentrations (25 µg/ml) development continued, but at much slower rates. This effect could be reversed by removing the embryos from Con A.

Moran (1974b) reported that *in vitro* migration of neural

crest cells from *Ambystoma mexicanum* was inhibited by Con A. Neural crest cells *in vivo* display extensive migratory capabilities and differentiate to form a variety of cell types, for example pigment cells, mesenchymal cells and sympathetic ganglia. *In vitro* cultures of amphibian neural crest cells undergo two distinct phases. Firstly, the migratory phase, in which rounded cells detach from the explant and disperse from it in an ameboid fashion. Following the cessation of migratory movements, a differentiation phase begins. The cells become visually differentiated, with a reduction in yolk content, and become dendritic and/or pigmented. Moran (1974b) reported that Con A inhibited migration in a concentration-dependent manner. At low concentrations of lectin (12.5 µg/ml), the cells which were capable of migrating were unable to differentiate. Treatment with Con A after migration resulted in a reversal of differentiating cells, with slightly specialized cells returning to rounded form, while cells which had completed differentiation to the stellate and/or pigmented form did not undergo this reversal. Moran (1974b) concluded that upon the cessation of migration and completion of differentiation, the effects of Con A become diminished.

Lee (1976) showed that Con A at low dosages (12-24 µg/ml) could inhibit closure of the neural tube in explanted chick embryos (stages 4-7, Hamburger and Hamilton, 1951) by inhibiting interkinetic nuclear migration. Erythropoiesis, migration of precardiac cells to form tubular heart, regression of the primitive streak or somite formation were usually not affected. In control experiments, cells labelled with tritiated thymidine were initially located at the outer

zone of the neuroepithelium, and then these labelled cells migrated to the neurocoele for mitosis. These cells then returned to the outer zone to start new DNA synthesis in preparation for the next division. Lee found that this migration was inhibited in Con A-treated embryos, where nuclei were found to divide throughout the neuroepithelium. It has been suggested that this interkinetic nuclear migration is important for closure of the chick neural tube (Messier and Auclair, 1974).

b) *Distribution of CABS and Agglutinability*

Con A has been used to monitor cell surface changes in a number of developmental or differentiating systems. The pattern which has emerged from these studies indicates that differences, usually a reduction in CABS, occur in the cell surface as differentiation proceeds. There are a wide range of possible explanations which may account for the differences in Con A binding. The CABS on the cell surface may be altered in such a way as to be no longer available for binding, they may be lost through a turnover in membrane, or alternately the distribution of the sites may be altered so they are no longer capable of interacting in the same manner.

During fertilization and maturation of eggs, a change in the amount or distribution of CABS has been found in the *Ascidia* egg and mouse egg. O'Dell *et al.* (1973) reported that CABS were undetectable in the unfertilized and newly fertilized *Ascidia* egg, but they made a sudden appearance at the surface of the egg at the time of maturation. It was found that a two- to four-fold increase in Con A was bound at the time of expulsion of the second polar body. This level of binding

remained relatively constant up to the four-cell stage. As there is no significant change in cell surface area, this is believed to represent an increase in the numbers of CABS (O'Dell *et al.*, 1973). Johnson *et al.* (1975) reported an uneven distribution of FITC-Con A over the surface membrane of unfertilized mouse eggs. An area of weak or absent staining which covered approximately one-fifth of the egg's surface was found. This was associated with the underlying second metaphase body. Newly fertilized eggs at pronuclear and earlier stages displayed a smooth staining pattern. The membrane covering the midbody connection between the polar body and the egg was intensively stained, but the polar body was either always negative or stained in a small cup-shaped region which joined the midbody. It was suggested that the mosaicism of CABS displayed at the cell surface might reflect the underlying intracellular organization. The molecular basis for the mosaicism could be due to either a masking of Con A residues in the negatively staining region or exclusion of CABS from this region. It was experimentally determined that masking was not by neuraminidase-sensitive material (Johnson *et al.*, 1975).

As well as at these relatively early stages of development, studies done on a wide variety of invertebrates and vertebrates indicated that there are alterations in the binding of Con A as development proceeds. These studies have used FITC-Con A to map the distribution of CABS over the cell surface or have used agglutination assays. It has been assumed that agglutination assays are indicative of the presence of CABS on the cell surface. Generally, it has been found that as cells become more differentiated, they become less susceptible to agglutination

by Con A.

Weeks (1973) has shown that Con A agglutinates cells from the slime mold (*Dictyostelium discoideum*) in a differential manner. During the early stages of differentiation, free living myxamoebae aggregate to form a multicellular organism, which then forms a fruiting body. It was found that as cells passed from the phase of exponential growth to stationary growth, the susceptibility to agglutination gradually decreased (Weeks, 1973). A similar decrease in agglutinability was reported as the cells passed from the vegetative amoebae to the aggregated form (Weeks and Weeks, 1975).

Krach *et al.* (1974) provided evidence for the age-dependent differential agglutinability of sea urchin embryonic cells. They reported that dissociated day-old embryos displayed greater agglutinability than 7-day old dissociated embryos. Further studies done by this group (Roberson and Oppenheimer, 1975) indicated that in the case of dissociated 32/64 cell sea urchin embryos, variability in Con A-mediated agglutination exists with respect to cell type. Dissociated embryos were separated by Ficoll gradients into micromeres and a combined mixture of mesomeres and macromeres. It was reported that the micromeres were significantly more agglutinable than the other cell populations. From this evidence and previous work (Krach *et al.*, 1974), it was suggested that the micromeres were responsible for most of the agglutination of dissociated 32/64 stage sea urchin embryos. Micromeres are presumptive mesenchyme cells and undergo extensive migratory cell movements during gastrulation. As these micromeres differentiated with increasing embryonic age, their migratory ability

and agglutinability decreased (Krach *et al.*, 1974).

Roberson and Oppenheimer (1975) reported that dissociated sea urchin embryonic cells treated with colchicine, a microtubule-disrupting agent, showed an increase in Con A-mediated agglutinability. Recently, microtubule and microfilament systems have been implicated in the control of mobility and topographical distribution of receptor sites (for recent review see Nicolson, 1976). Roberson and Oppenheimer (1975) suggested that the basis for decreased agglutinability with increasing age may be due to any of the following: decreased numbers of CABS present, decreased accessibility of these sites for interaction with lectin, randomization of CABS or reduced receptor mobility. It was felt that the results indicated that colchicine-sensitive components may be responsible for anchoring CABS in a pattern which inhibited the mobility of these sites and hence decreased agglutination.

In further studies, the pattern of FITC-Con A was investigated on the surface of micromeres, macromeres and mesomeres (Roberson *et al.*, 1975). If all three populations all cells were treated with FITC-Con A after fixation, the distribution of CABS appeared to be random. However, if the cells were treated with FITC-Con A prior to fixation, it was found that 95% of the micromeres displayed FITC-Con A in a capped or clustered configuration, while the distribution of FITC-Con A on the macromeres and mesomeres remained random. This evidence indicated that the CABS on the migratory cell population were more mobile and could diffuse more readily in a lateral manner through the lipid bilayer of the cell membrane. This greater mobility may be due to a greater intrinsic fluidity of the lipid bilayer, structural modification

of CABS, or alterations in the structure of microfilaments or microtubules attached to the inner membrane surface, which may control the mobility of glycoproteins or proteins within the membrane.

Fluorescent Con A has been used to map CABS in whole fixed *Xenopus* embryos (O'Dell *et al.*, 1974). In unfertilized or fertilized eggs, or during cleavage stages, no generalized binding was found, but there was binding at the margins of cleavage cells. In later stages, binding was observed over the surfaces of some cells, specifically the cells within the blastopore lip and within the medullary plate. During gastrulation, intense binding was observed in cells concentrated in the marginal zone of the blastopore lip and at the yolk plug stage, fluorescence cells formed a ring around the yolk plug. During neurulation, the presumptive neural plate was labelled, and this label later became concentrated in the neural ridges. When the neural folds fused, a narrow line of fluorescence marked the closure of the tube. O'Dell *et al.* (1974) proposed that the most obvious correlation shown by the surface binding patterns in these experiments was that cells undergoing morphogenetic movement show a high affinity for the lectin. Con A treatment of living *Xenopus* did not interfere with development. It was also suggested alternatively that this binding pattern may result from FITC-Con A labelling intracellular material attached to cells, or that the label may be detecting new surface material which was coincidently attached to migrating cells.

Johnson (1975) incubated FITC-Con A with EDTA-dissociated cells from blastula and gastrula *Rana* species. He reported that blastula cells showed a homogeneous distribution of bound FITC-Con A

after 30 minutes incubation, but that gastrula stage cells showed a clustered or capped appearance of bound FITC-Con A. Con A-induced redistribution of CABS on the cell surface of gastrula cells was inhibited by fixation or incubation at 8°C. Furthermore, the pattern after 5 minutes incubation showed a uniform distribution, but with more time became capped or clustered in unfixed gastrula cells. Johnson suggested that these results indicate that there was an increased mobility of CABS as *Rana* embryos develop from blastula to gastrula stages. He did not find any difference in the amount bound or the pattern of binding in cells taken from different regions of the gastrula.

Lectin-mediated agglutination has been studied in embryonic chick and human cells. Weiser (1972) reported that Con A agglutinated cells from human fetal intestine but not adult intestine. Additionally, both stage 1 and stage 4-5 chick embryos dissociated with EDTA are agglutinated by Con A (Zalik and Cook, 1976). Trypsin or EDTA-dissociated chick liver and embryonic retina are also agglutinated by Con A (Moscona, 1971). Kleinchuster and Moscona (1972) demonstrated that agglutination of dissociated 10-day embryonic chick neural retina cells differed with respect to stage of differentiation. It was found that agglutination was greatest with cells from 8-day embryos and this decreased with increasing embryonic age to the smallest level of agglutination at 20-day cells. Brief trypsin treatment of 20-day cells restored agglutinability with Con A. Kleinschuster and Moscona (1972) interpreted this to mean that with increasing differentiation CABS become masked or sheltered by trypsin-sensitive material. This masking

of receptor sites could be due to the synthesis of specific moieties produced at particular stages in differentiation, to configurational changes in the receptors, or to architectural changes within the cell surface which position CABS more internally.

An alternative explanation for the effect of trypsin may be the spontaneous rearrangement of membrane components as proposed by Nicolson (1972), which could tend to increase the mobility of the CABS. McDonough and Lilien (1975a) examined the lectin receptor mobility in cells dissociated from embryonic chick neural retina. They dissociated retinas from 10-day chicks by a variety of means and followed the receptor mobility by FITC-Con A. Cells obtained from neural retinas by trypsin treatment and mechanical dissociation were able to redistribute their CABS into caps both in the presence or absence of Con A. However, cells prepared by EDTA or incubation in calcium and magnesium-free solution and mechanical dissociation could not redistribute their CABS into caps. Cells in intact tissues were also unable to show the redistribution pattern.

If cells prepared by trypsin treatment were allowed to recover for 4 hours in culture, prior to incubation with Con A, they did not exhibit a redistribution of CABS into caps. McDonough and Lilien (1975a) suggested that as these cells originate from a tissue which is undergoing differentiation, the repair processes may return the cell surface to the condition in which it exists *in situ*, that is, it would be more similar to intact tissue. As it was found in this study that intact tissue could not redistribute CABS, these workers felt on this basis that membrane fluidity may be important during the

early processes of tissue formation.

In further studies, McDonough and Lilien (1975b) reported that the binding of factors isolated from tissues, which appeared to mediate recognition and adhesion, inhibited in a tissue-specific manner the redistribution of lectin receptors in the plane of the membrane. It was found that aggregation-promoting materials (APMs) could be isolated from retina and cerebral lobe of 10-day chick embryos which enhanced reaggregation of dissociated cells in a tissue-specific manner. These APMs were found to bind to specific terminal residues on the cell surface. The effect of this binding was to inhibit the redistribution of lectin receptors into caps. McDonough and Lilien (1975b) suggested that control of cell-surface topography of ligands specific for tissue type may be of fundamental importance to the processes of cell recognition and adhesion. The distribution of surface components important in recognition and adhesion may at least partially determine if stable cell contacts form between neighbouring cells. These ligands may then serve through their action at the cell surface to maintain the surface components in an appropriate configuration, allowing for the maintenance of stable cell contacts.

MATERIALS AND METHODS

1. *Preparation of Embryos*

Chick embryos were obtained from fertilized Leghorn and Hubbards' eggs in the following manner. The top of the egg was broken with forceps and the albumen drained. After cutting through the vitelline membrane the blastoderm was removed to a dish of Panett and Compton's saline. The embryo was floated off the egg-spoon and separated from the vitelline membrane and underlying yolk with fine forceps. It was then transferred to a second dish of fresh Panett and Compton's saline with a wide-mouthed pipette and stored at 4°C until fixation. Panett and Compton's saline was made up as follows: 48.4 g NaCl, 6.2 g KCl, 3.1 g $CaCl_2$, 5.1 g $MgCl_2.6H_2O$ and 2.42 g of Tris (Sigma, reagent grade Trizma, pH 7.5) were dissolved in 1 liter of distilled water. The pH of this concentrate was adjusted to 7.5 at room temperature and stored at 4°C. A ten-fold dilution was made and used as a final solution.

Incubation was carried out in a Brower incubator at 37°C. Staging was in accordance with Hamburger and Hamilton (1951) and was performed under a binocular dissecting microscope. Incubation periods of 6 hours yielded blastoderms of stage 1 to stage 2 and incubation periods of 22 hours yielded a majority of blastoderms of stages 4-5. Unincubated embryos will not be referred to as "stage 1", but as "unincubated". The anterior region of the blastoderm was marked by removing a wedge of tissue to facilitate orientation at the time of embedding.

2. *Electron Microscopy*

a) *Transmission Electron Microscopy*

Two buffer systems were used: a 0.1 M phosphate buffer (Sørensen's), pH 7.4, and a 0.1 M cacodylate buffer, pH 7.4. It was found that the cacodylate buffer was more stable and was therefore routinely used in later preparations. The embryos were rinsed well with buffer after storage in Panett and Compton's saline and then fixed in 3.5% glutaraldehyde (Ladd) for 2 hours at 4°C. They were then rinsed four times with a buffer for a total of 40 minutes. Post-fixation was carried out for 1 hour at 4°C in 1% osmium tetroxide and after a thorough buffer rinse, the embryos were dehydrated in a graded series of ethanol solutions, followed by propylene oxide and embedded in araldite (Poly Sciences).

Stage 1 embryos were routinely sectioned in two areas: anteriorly and posteriorly. Unincubated embryos could not be oriented with any assurance as to an anterior-posterior axis and were sectioned in two areas at random. Stage 5 embryos were sectioned in an anterior region, either anterior to Hensen's node or through Hensen's node, and then through the anterior third of the primitive streak. As well, some embryos were sectioned in a third region, in the posterior two-thirds of the primitive streak (Fig. 2). Normally most blocks were trimmed in the midline region of the embryo. During the trimming process, thick 1 μ sections were cut and stained with methylene blue and azure blue (a 1:1 mixture of 1% azure blue in distilled water and 1% methylene blue in 1% borax solution) for light microscopy. This ensured that appropriate areas of the embryo were sectioned.

Fig. 2. Scanning electron micrograph of stage 4-5 embryo, seen from the dorsal surface of the epiblast. Sections were routinely taken through the anterior region, anterior one-third of the primitive streak. HN, Hensen's Node; PS, primitive streak. Arrows indicate from where the sections were taken.
x420

Silver coloured sections were picked up on formvar-coated copper grids and stained with 5% aqueous uranyl acetate and lead citrate. All grids with sections taken from cytochemical experiments were observed unstained. The grids were examined with a Philips 300 electron microscope.

b) *Scanning Electron Microscopy*

Embryos were rinsed with 0.1 M cacodylate buffer, pH 7.4, and fixed for 2 hours in 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, at 22°C. They were then rinsed in distilled water and dehydrated in a graded series of acetone solutions. The embryos were dried in a critical point drying apparatus (Polaron Equipment Ltd.), mounted on stubs, coated with a gold conducting layer, and examined in a stereoscan S4 electron microscope (Kent Cambridge Ltd.).

3. *Experimental Procedures*

Four cytochemical techniques were used to label Concanavalin A bindings sites (CABS). The first three rendered the CABS electron dense.

a. A modification of the method of Bernhard and Avrameas (1971) using Graham and Karnovsky's (1966) horseradish peroxidase and diaminobenzidine technique.

b. Ferritin conjugated covalently to Concanavalin A by the method of Nicolson and Singer (1971) (purchased from Calbiochem).

c) The technique of Martin and Spicer (1974) using iron dextran to bind to Concanavalin A.

d. Fluorescein isothiocyanate-labelled Concanavalin A was used in conjunction with light microscopy.

a) *Concanavalin A-Horseradish Peroxidase-Diaminobenzidine*

Embryos were thoroughly rinsed with 0.1 M cacodylate buffer, pH 7.4, after storage in Panett and Compton's saline and fixed for 2 hours in 2.5% cacodylate buffered glutaraldehyde. The tissue was then incubated in 100 µg/ml Concanavalin A (Con A, Sigma) in cacodylate buffer, pH 7.2, for 2-5 hours. Control embryos were incubated in Con A plus 0.5 M α-methyl-D-mannoside (αMM, Sigma Grade III). The blastoderms were then rinsed three times for a total of 1 hour with buffer and incubated in 50 µg/ml horseradish peroxidase (HRP, Sigma Type VI) in cacodylate buffer for 30 minutes to 2 hours. Control embryos were incubated in HRP solution which contained 0.5 M αMM. Rinsing was carried out six times for a total of 2 hours and embryos were then incubated in a saturated aqueous solution of 3,3' diaminobenzidine hydrochloride (DAB, J.T. Baker analyzed reagent) and 0.01% hydrogen peroxide in 0.5 M Tris-HCl buffer, pH 7.6, for 15-30 minutes. This was followed by a thorough rinsing in buffer. All of the above was carried out at 22°C. The embryos were post-fixed in 1% cacodylate buffered osmium tetroxide for 1 hour at 4°C. After a thorough rinse, the material was dehydrated and embedded in araldite.

This cytochemical procedure was used on unincubated, stage 1 and stage 4-5 embryos as well as stage 1 embryos which had been dissected into hypoblast and epiblast fragments. This dissection was performed by securing each blastoderm in agar plates and gently teasing

off the hypoblast with tungsten needles. Very small fragments of hypoblast were obtained and material was pooled and spun down in a microfuge (Beckman). Large pieces of epiblast were carried through the cytochemical procedure individually.

As a control against subjective error, grids from several different cytochemical procedures were coded and given to an unbiased observer. The grids were scored as either experimental or control tissue according to the intensity of the reaction product.

Additional control experiments were carried out as follows. A second sugar, α-methyl-D-glucoside (αMG) was used instead of αMM to determine the relative effectiveness of each as a hapten inhibitor. A 0.5 M concentration of αMG was added to the Con A and HRP control solutions in the same manner as αMM.

Further controls were done in the absence of Con A to eliminate the possibility of non-specific binding of reaction to product to the cell surface. The first set of glutaraldehyde-fixed blastoderms was incubated directly with 50 μg/ml HRP for 30 minutes, rinsed and placed in a saturated solution of DAB with 0.01% hydrogen peroxide in Tris-HCl buffer for 15 minutes. A second set of embryos was incubated in saturated DAB in Tris-HCl buffer for 15 minutes and then incubated with 3×10^{-3} M potassium ferricyanide for 5 minutes. Since potassium ferricyanide readily oxidizes DAB and thus mimics the effect of hydrogen peroxide, this control was to determine whether artificial adsorption of DAB or its oxidation product occurred. A third set of blastoderms was incubated in saturated DAB and 0.01% hydrogen peroxide in Tris-HCl buffer. All of the embryos in these

experiments were post-fixed for 1 hour in 1% osmium tetroxide.

b) *Ferritin-Concanavalin A*

Embryos which were stored in Panett and Compton's saline were rinsed well with 0.1 M phosphate buffer, pH 7.4, and fixed for 2 hours in 2.5% phosphate buffered glutaraldehyde (Ladd) at 22°C. After a thorough rinse with phosphate buffer, the material was incubated in approximately 100 µg/ml Con A-ferritin (Calbiochem, A grade) in phosphate buffer, pH 6.8, for 1 hour. Control embryos were incubated in Con A-ferritin plus 0.5 M αMM (Sigma Grade III). The tissue was then rinsed six times for a total of 2 hours with phosphate buffer. This was followed by post-fixation in 1% osmium tetroxide for 1 hour at 4°C. The blastoderms were dehydrated in an ethanol series and embedded in araldite.

c) *Iron Dextran-Concanavalin A*

Embryos were rinsed with 0.1 M cacodylate buffer, pH 7.4, and fixed for 2 hours in 2.5% glutaraldehyde at 22°C. After a thorough rinse the experimental embryos were incubated for 5 hours in 100 µg/ml Con A, pH 7.2, and the control embryos were incubated in Con A and 0.5 M αMM. Following three rinses for a total of 60 minutes, the embryos were incubated for 2 hours in 10 mg/ml iron dextran (Imferon, Fisons Canada Ltd.) in 0.1 M cacodylate buffer, pH 7.4, at 22°C. After three rinses for a total of 60 minutes the embryos were post-fixed in 1% osmium tetroxide, dehydrated and embedded in araldite.

d) *Fluorescein Isothiocyanate-Concanavalin A (FITC-Con A)*

Stage 5 embryos were rinsed thoroughly with 0.1 M phosphate buffer, pH 7.4, and fixed for 30 minutes in 2.5% phosphate buffered glutaraldehyde at 22°C. The blastoderms were then incubated for 15-30 minutes in 500 µg/ml FITC-Con A (Miles-Yeda) in 0.1 M phosphate buffer, pH 7.2. Control embryos were incubated in FITC-Con A and 1M αMM. The tissue was dehydrated in an ethanol series, cleared in creosote and observed with UV transmitted light (Zeiss photomicroscope). Two exciter filters, a UG1 and a BG38, were used.

RESULTS

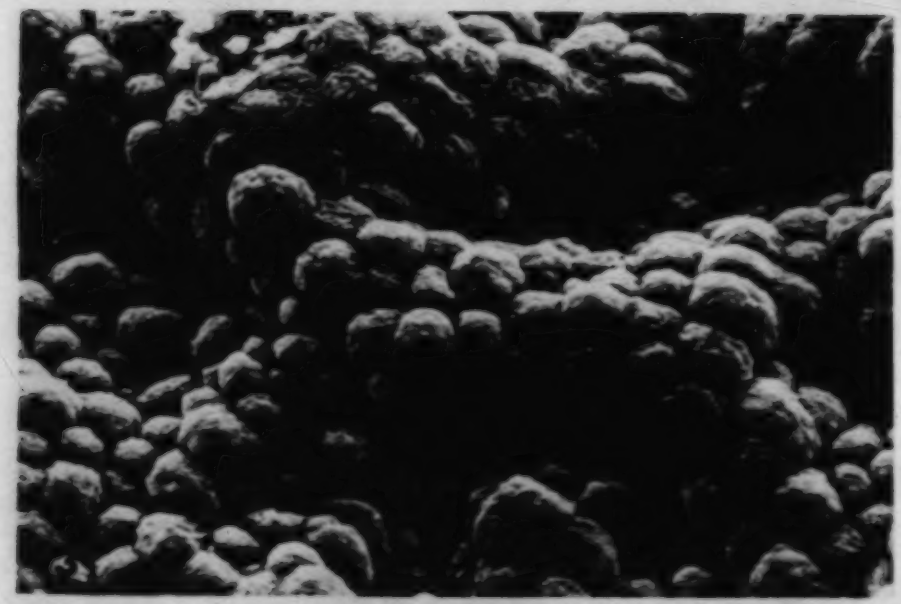
1. *Light and Scanning Electron Microscopy: Description of Areas Sectioned*

The dorsal surface of the epiblast is characterized by junctional complexes between the epiblast cells, composed of focal tight junctions and incipient desmosomes (Fig. 32). This allowed orientation of surfaces with respect to dorsal or ventral aspects as no comparable complex existed at other surfaces. In anterior regions of unincubated or stage 1 embryos, the epiblast consisted of columnar-shaped cells with the hypoblast layer beneath it. The extent to which the hypoblast formed a complete coherent layer varied from a few apparently isolated cells (Fig. 7) to larger groups of adhering cells (Fig. 8). In posterior regions, the hypoblast generally formed a complete coherent single layer of flattened cells (Fig. 8). When viewed with the scanning electron microscope, the dorsal surface of the epiblast is seen to be characterized by the presence of microvilli (Fig. 4). The surface of the epiblast is also characterized by depressions, which give the surface an undulating appearance (Fig. 3).

Stage 4-5 embryos sectioned in anterior regions usually displayed a complete hypoblast layer overlaid by the columnar epiblast (Fig. 9). The hypoblast in some areas appeared to be composed of more than a single layer of cells. Sections taken from Hensen's node region displayed a fused thickened layer composed of both epiblast and hypoblast (Fig. 10), but immediately to the periphery the two layers separated into distinct epiblast and hypoblast. The dorsal surface of

Fig. 3 Scanning electron micrograph of a stage 1 embryo; view of dorsal surface of the epiblast. x10,500

Fig. 4 Same as Fig. 3 showing microvilli, MV. x105,000



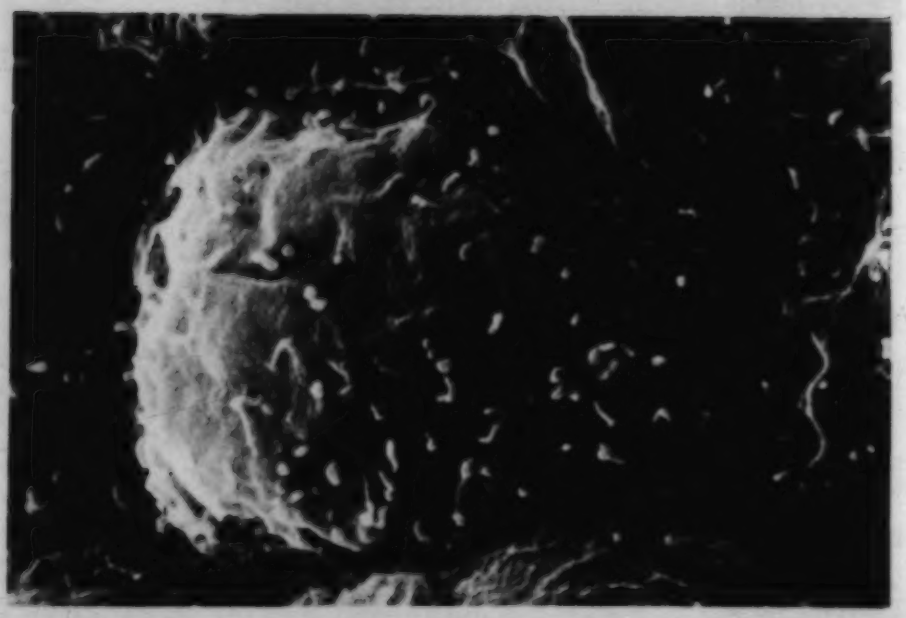
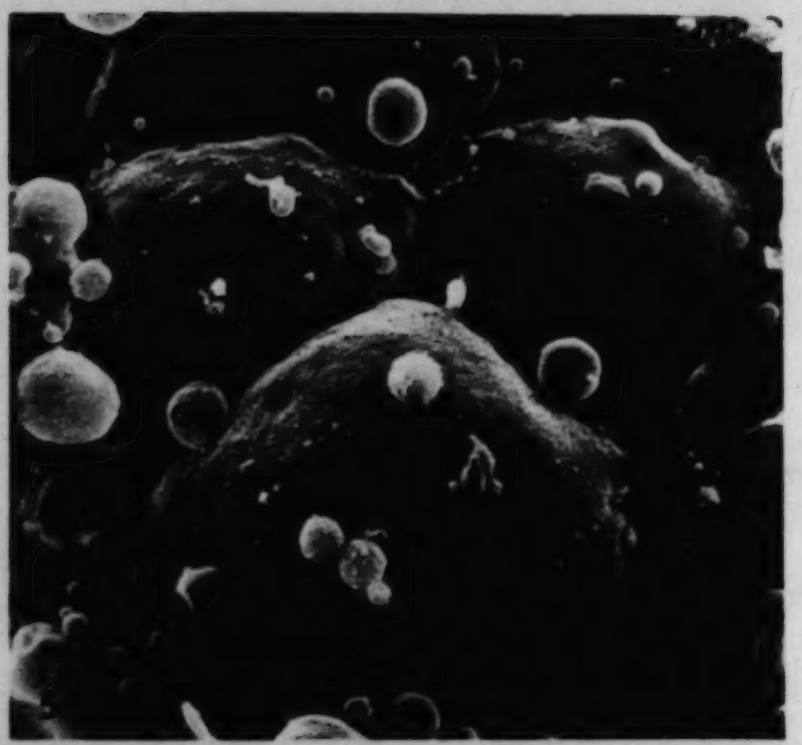


Fig. 5 Scanning electron micrograph of a stage 4-5 embryo in the anterior one-third of the primitive streak region. These cells were found in the wall of the primitive groove. GP, globular projections. x105,000

Fig. 6 Scanning electron micrograph of stage 4-5 approximately in the midpoint of the primitive streak. GP, globular projections; C, cilium. x210,000



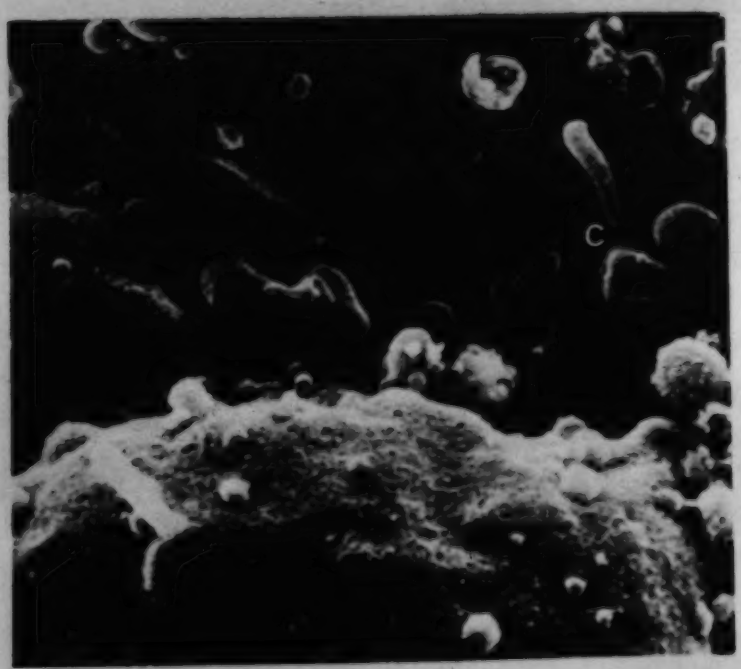
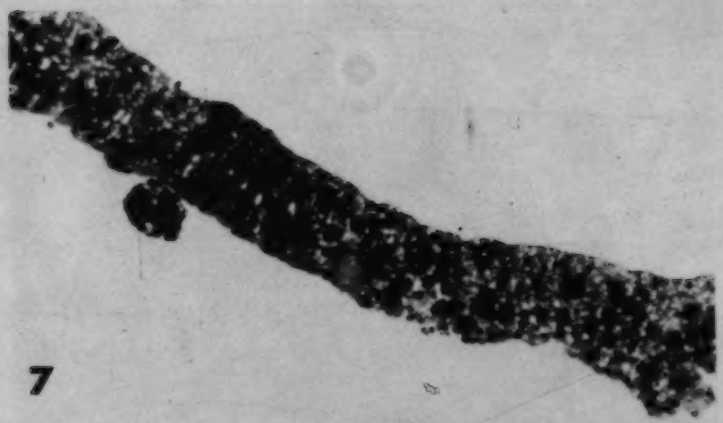

C

Fig. 7 Light micrograph of a stage 1 embryo sectioned in the anterior portion of the embryo showing incomplete hypoblast (H). x540

Fig. 8 Light micrograph of a stage 1 embryo sectioned in the posterior region with complete hypoblast (H). x330



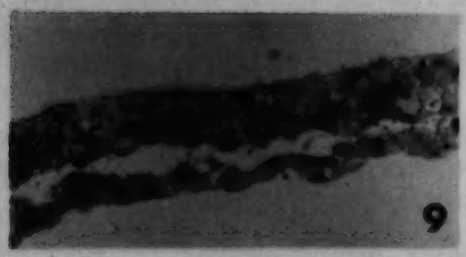
7

8

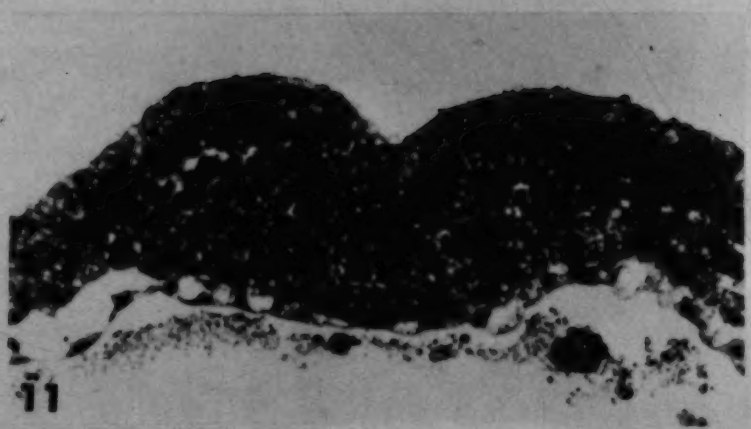
Fig. 9 Light micrograph of a stage 4-5 embryo sectioned in the anterior region. x330

Fig. 10 Light micrograph of a stage 4-5 embryo sectioned through Hensen's node showing fused thickened layer. x330

Fig. 11 Light micrograph of a stage 4-5 embryo sectioned through the primitive streak. x330


9

10


11

Hensen's node was referred to as the dorsal surface of the epiblast and the ventral surface as the ventral surface of the hypoblast. Three distinct layers again become evident as one moves from the midpoint of the embryo towards the periphery. Immediately posterior to the node, the primitive streak is seen (Fig. 11). In this region, the epiblast forms a groove, the base of which is continuous with the hypoblast. As one moves peripherally, three separate layers become evident: epiblast (presumptive ectoderm), mesoblast (presumptive mesoderm) and hypoblast (presumptive endoderm).

In stage 4-5 embryos, some changes in surface morphology have occurred. On the dorsal surface of the epiblast, at the junction of two cells, a cell process is usually seen projecting from one cell to the other (Figs. 56 and 57). Microvilli are much less common, particularly in areas immediately adjacent to the primitive streak, and instead globular projections are seen (Figs. 5 and 6). As well, each epiblast cell is now equipped with a cilium (Figs. 6 and 50) and in the region of the primitive streak the groove itself contained cell debris (Fig. 29) (for a complete study of surface morphology by scanning and transmission electron microscopy see Bancroft and Bellairs, 1974; Jacob *et al.*, 1974).

2. *Concanavalin A-Horseradish Peroxidase-Diaminobenzidine*

This cytochemical procedure was performed on unincubated embryos, stage 1 (incubated 6 hours) and stage 4-5 embryos (incubated 22 hours). A total of five unincubated embryos, eleven stage 1 and twenty-one stage 4-5 embryos were used. It was found that unincubated

embryos could not be oriented with any assurance as to an anterior-posterior axis, so an incubation period of 6 hours was used to facilitate orientation by means of the embryonic shield.

Con A-HRP-DAB produced a diffuse dense reaction product which did not penetrate the plasma membrane. This cytochemical method is not easily quantitated, and reaction product was evaluated arbitrarily on the basis of density or thickness of the layer of reaction product and whether it displayed a clumped or even pattern of binding. Results are summarized in Table 1. The scoring of the reaction product was as follows: 0, no reaction product; + (for example see Fig. 14), a sparsely scattered thin layer; ++ (for example see Fig. 15), a more dense layer in which most of the membrane has reaction product; and +++ (for example see Fig. 13), a very thick deposit over the entire membrane. In some sections, the membrane displayed regions which could possibly fit into two categories, so some overlap occurred, but an attempt was made to assign, as objectively as possible, a category which represented the majority of the area of cell surface scanned. It was found that penetration by the reaction product through the junctional complexes to stain lateral surfaces of epiblast cells did not occur. However, if the hypoblast was incomplete or damaged, some reaction product was found penetrating from the ventral surface of the epiblast towards the dorsal surface of the epiblast (Fig. 13). No penetration of reaction product was found in stage 4-5 into the cavity between the epiblast and hypoblast. The symbol Ø is used in Table 1 to indicate that although no reaction product is seen on these surfaces, this may reflect the inaccessibility of the surfaces to the cytochemical

Table 1

Summary of Results of Incubation With

Conconavalin A-Horseradish Peroxidase-Diaminobenzidine

Unincubated and Stage 1			Stage 4-5		
	Anterior	Posterior		Anterior	Primitive Streak
DE	++	++	DE	++	++, either ⌐¬ or ⌐⌐
VE	+++	∅	VE	0 and ∅	∅
DH	0 → +*	∅	DH	0 and ∅	∅
VH	+ → ++*	+ → ++	VH	0	0

See text for evaluation procedure and explanation of symbols.

* ○ - pattern observed on single hypoblast cells.

DE - dorsal surface of the epiblast

VE - ventral surface of the epiblast

DH - dorsal surface of the hypoblast

VH - ventral surface of the hypoblast

procedure. For the same reason, sections through the posterior of stage 1 embryos (which have a complete hypoblast) were scored as "Ø" for the dorsal surface of the hypoblast and ventral surface of the epiblast.

Cytochemical controls were performed which employed αMM as a hapten inhibitor. The majority of the embryos treated in this way showed no reaction product (Figs. 32, 33, 34 and 35). However, in all experiments some of these control embryos displayed a thin uneven pattern of reaction product, usually on the dorsal surface of the epiblast. In the series of control experiments done in the absence of Con A, no binding of HRP or DAB was found. Early experiments had determined that by decreasing the concentration of Con A and the incubation time with Con A and by increasing the rinsing time after incubation with HRP, a greater number of control embryos showed no binding of reaction product. Therefore, the occasional binding seen in some controls was considered non-specific.

Although within each experiment some variability existed between embryos with respect to corresponding surfaces, the following pattern resulted (Table 1). In stage 1 and in unincubated embryos all surfaces showed staining except where a complete layer of hypoblast was found in the posterior regions of the embryo. In these areas no reaction product was found on the ventral surface of the epiblast (Fig. 18) or the dorsal surface of the hypoblast (Fig. 19). Except for this, no differences were found between embryos sectioned in anterior and posterior regions. In most cases the dorsal surface of the epiblast showed a thick clumped pattern (++) (Figs. 12 and 17),

although some embryos showed a light even pattern. The ventral surface of the epiblast, if stained, showed a very dense clumped pattern (+++) which in comparison to the dorsal surface of the epiblast was much more dense (Figs. 12 and 13). If the hypoblast was not complete, individual hypoblast cells showed a non-uniform distribution so that the ventral surface of individual cells possessed denser deposits than the dorsal surface of the same cell (Fig. 16). If the hypoblast was complete, the dorsal surface was unstained (Ø) (Fig. 19), and the ventral surface was always stained but the pattern varied from a dense clumped layer (++) to a thin more even pattern (+) (Figs. 20 and 21).

Stage 4-5 embryos displayed binding on the dorsal surface of the epiblast in the anterior regions (++) (Fig. 22). The pattern was heavy and clumped, although there was some variation with some embryos showing a more even continuous pattern. In some anterior regions the hypoblast was not yet complete, however no reaction product was found on the ventral surface of the epiblast or the dorsal surface of the hypoblast, regardless of whether or not the hypoblast was a complete layer (Ø) (Figs. 23 and 24). The ventral surface of the hypoblast in anterior regions generally did not bind reaction product (Fig. 25), although out of eleven embryos two embryos did show a patchy pattern of reaction product on this surface. In areas taken from the region of the primitive streak, two distinct patterns of reaction product were found on the dorsal surface of the epiblast. In one experiment, five experimental embryos displayed one pattern, and in a second experiment all six experimental embryos displayed a second pattern. In the first pattern a dense clumped reaction product (++) was found on the dorsal

surface of the epiblast (Fig. 30) except as one approached the primitive streak. No reaction product was found on the walls nor through the base of the groove (⌒⌒) (Figs. 26 and 27). This loss of reaction product in the primitive groove was not seen in the second series of embryos and a dense clumped layer of reaction product was observed with no discontinuity (++ ⌒) (Figs. 28 and 29). No reaction product was observed in either the ventral surface of the epiblast or the dorsal surface of the hypoblast (Ø). The ventral surface of the hypoblast was generally free of reaction product (Fig. 31), although a few embryos did stain in a variable fashion from a light even to a patchy dense pattern.

Since in this cytochemical procedure some problems in penetration into the cavity between the epiblast and hypoblast occurred when a complete hypoblast was formed, an experiment was designed in which the complete hypoblast layer was stripped off. Both epiblast and hypoblast fragments were subjected to the cytochemical technique. The extremely small fragments of hypoblast necessitated pelleting, and it was found that orientation as to a dorsal or a ventral surface proved impossible, so no results are available for the hypoblast. The epiblast fragments showed a highly variable occurrence of reaction product on both surfaces, i.e., no reaction product on either surface; reaction product on only one of the two surfaces; reaction product on both surfaces. An example of no reaction product on the dorsal surface of the epiblast (0) with a dense clumped reaction layer (++) on the ventral surface of the epiblast is shown in Figs. 36 and 37. Control fragments demonstrated no binding of reaction product (Figs. 38 and 39).

Figs. 12 to 39 Transmission electron micrographs of embryos treated with Con A-HRP-DAB. Figs. 12 to 21 - stage 1 embryos. Figs. 22 to 31 - stage 4-5 embryos.

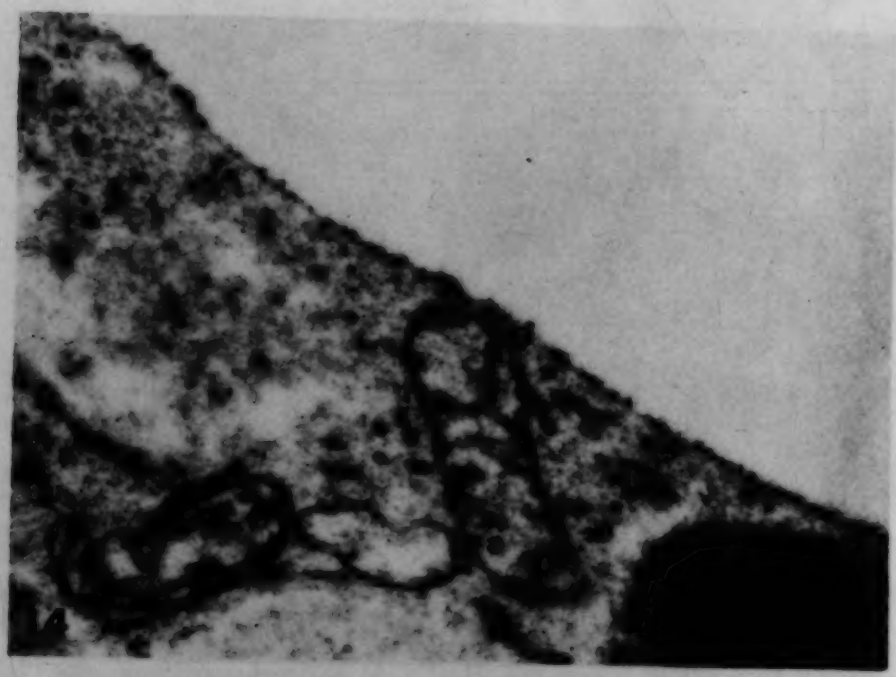
Fig. 12 Anterior region of the blastoderm, dorsal surface of the epiblast, ++; arrows indicate microvilli. x37,100

Fig. 13 Anterior region of the blastoderm, ventral surface of the epiblast, +++; reaction product penetrates towards the dorsal surface, hypoblast is incomplete. x37,100

13

Fig. 14 Anterior region of the blastoderm, dorsal surface of the hypoblast cell, + x37,100

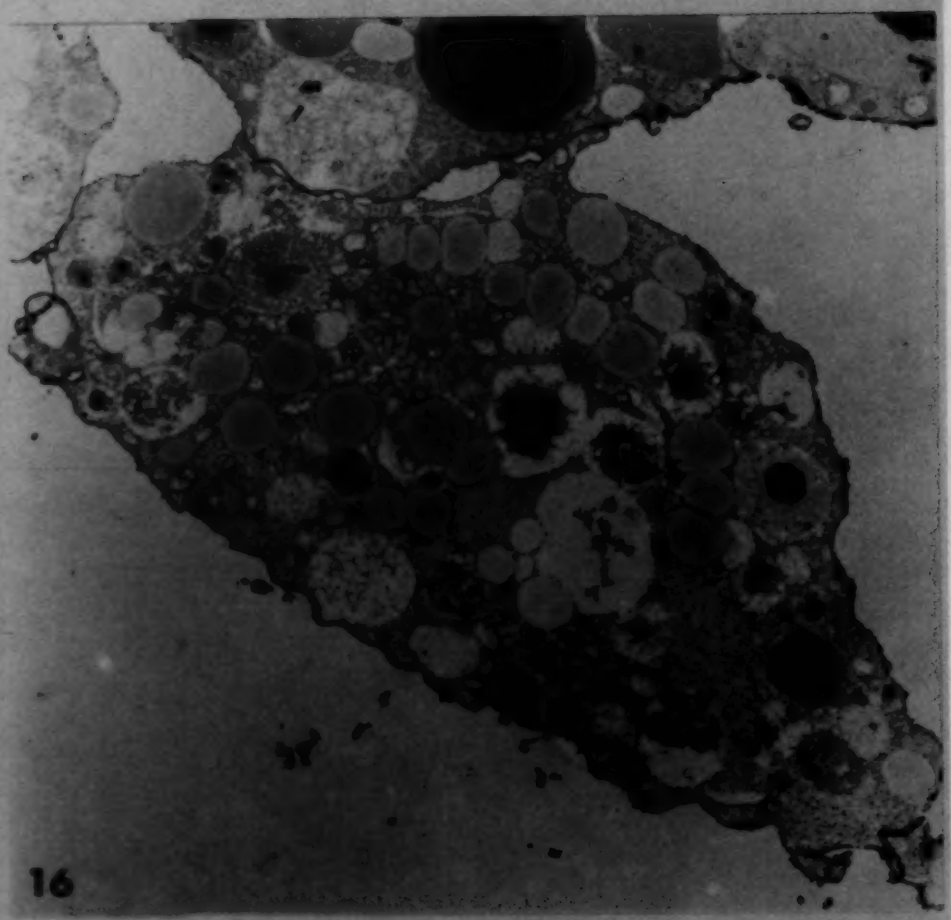
Fig. 15 Anterior region of the blastoderm, ventral surface of the hypoblast cell, ++ x37,100



14

15

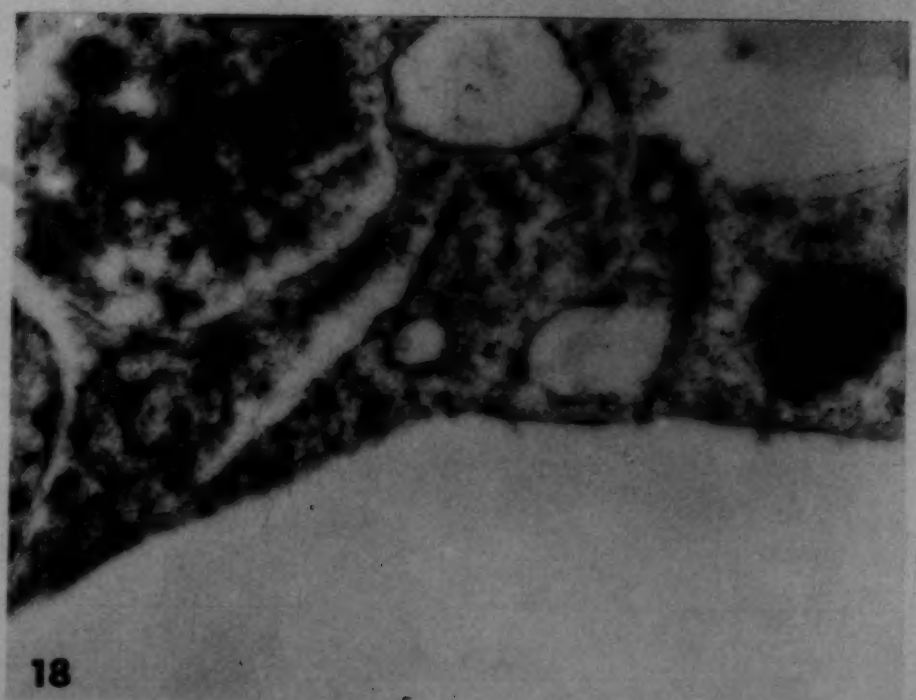
Fig. 16. Hypoblast cell from a region of incomplete hypoblast showing pattern of staining. See text.
x6,600



16

Fig. 17 Posterior regions of blastoderm, dorsal surface of the epiblast, ++; arrows indicate microvilli. x37,100

Fig. 18 Posterior region of the blastoderm, ventral surface of the epiblast with complete hypoblast, Ø x37,100

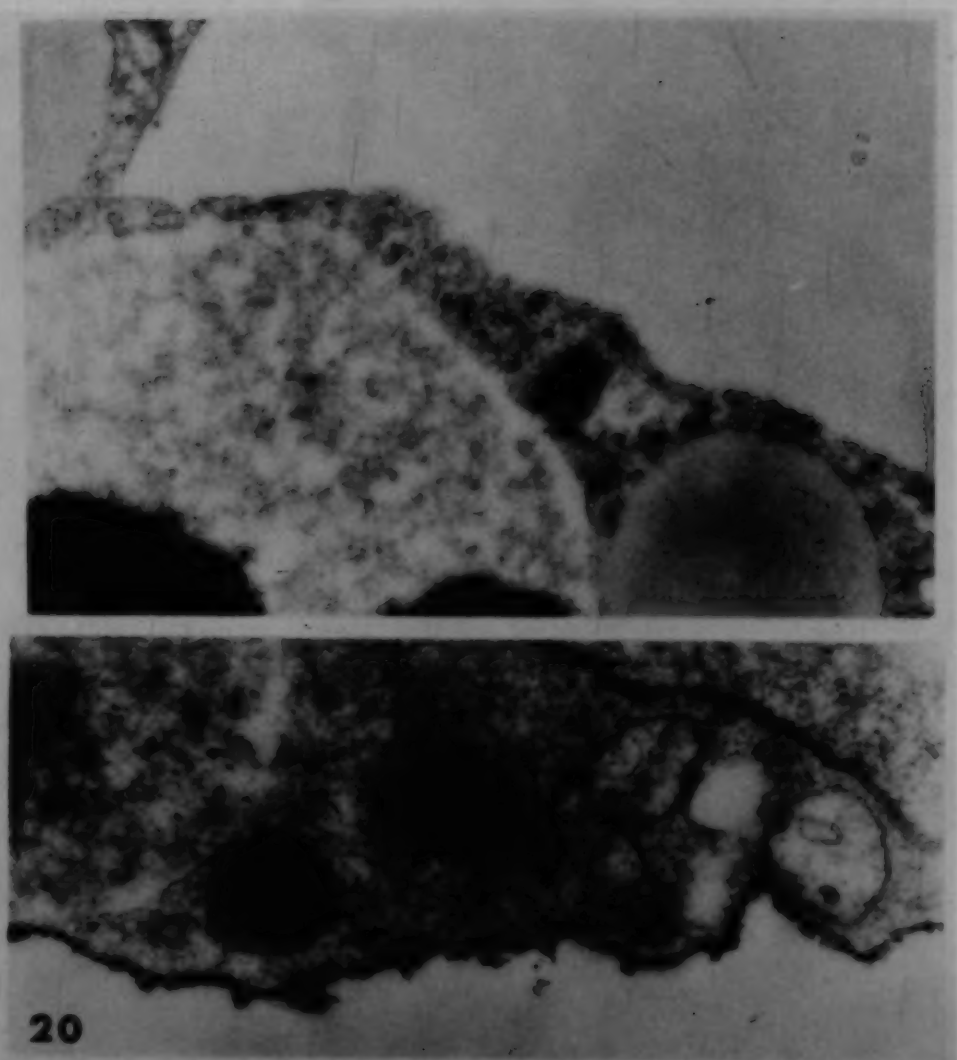


18

Fig. 19 Posterior region of the blastoderm, dorsal surface of the complete layer of the hypoblast, Ø x37,100

Fig. 20 Posterior region of the blastoderm, ventral surface of the complete hypoblast layer, ++, with clumped appearance. x37,100

Fig. 21 As in Fig. 20, +, with even appearance. x37,100



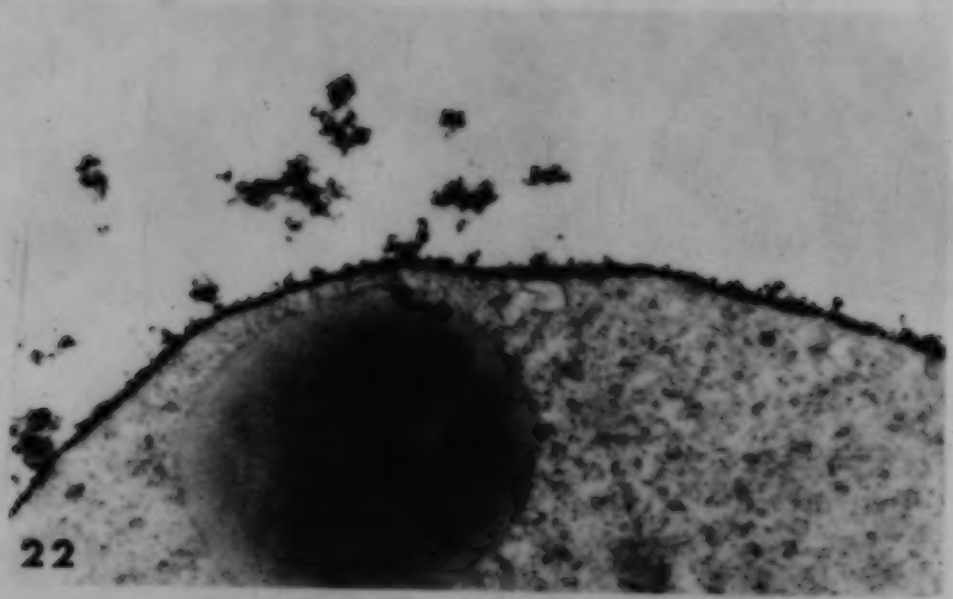
20

21

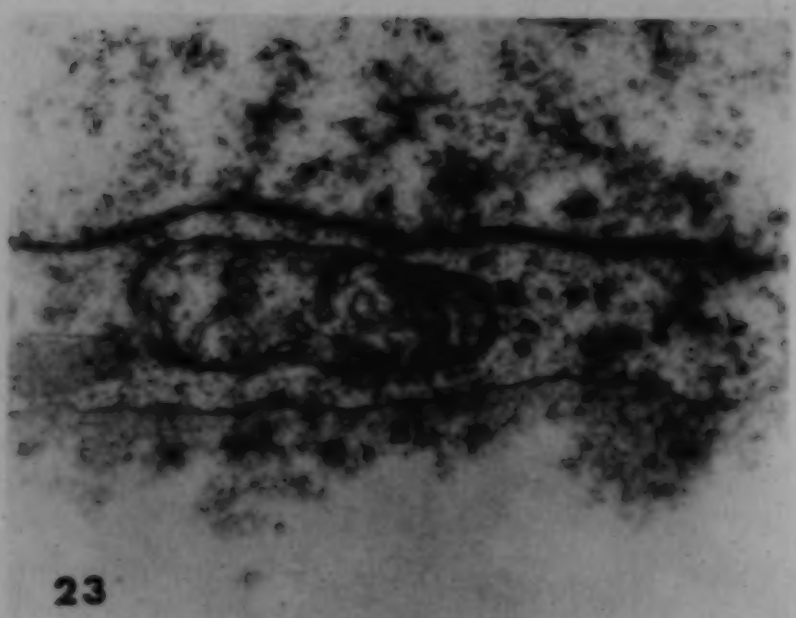
Figs. 22 to 31 Stage 4-5 embryos.

Fig. 22 Anterior region, dorsal surface of the epiblast, ++
x37,100

Fig. 23 Anterior region, ventral surface of the epiblast,
incomplete hypoblast, O or Ø; BL, basal lamina.
x37,100



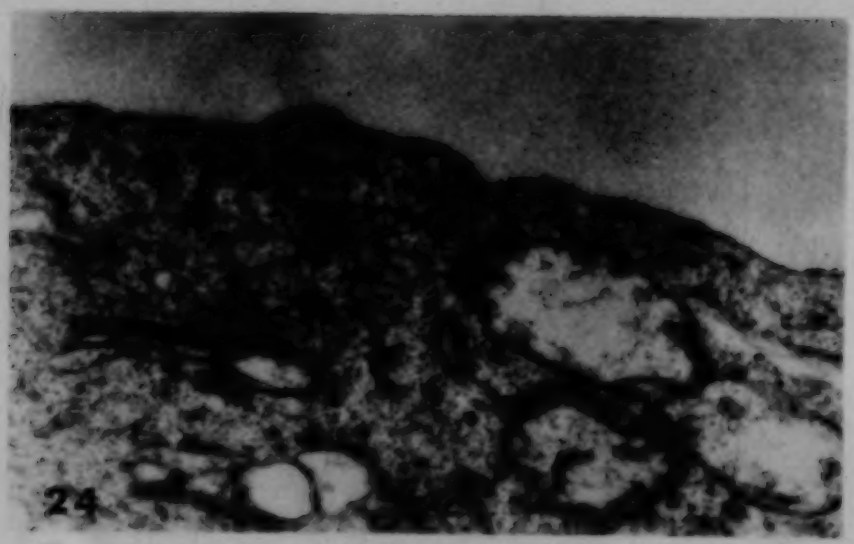
22



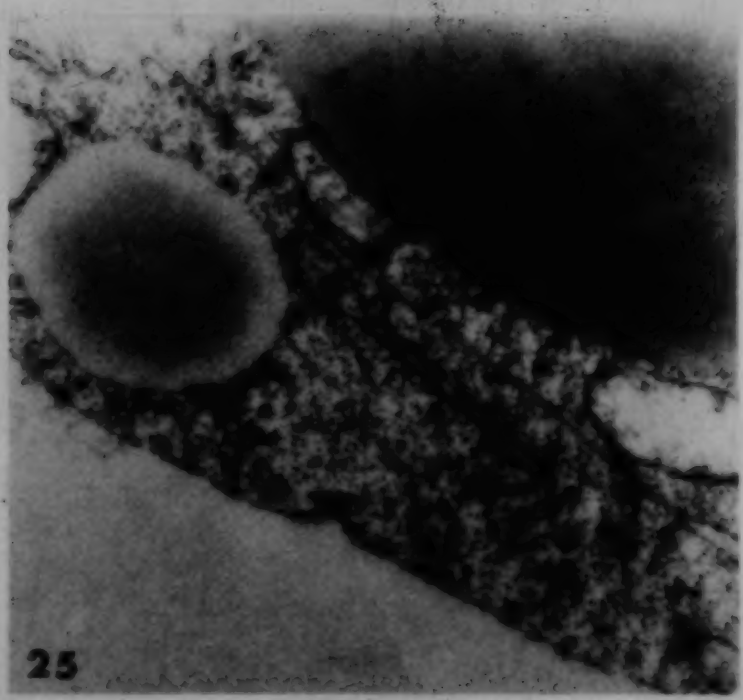
23

Fig. 24 Anterior region, dorsal surface of the complete hypoblast layer, O or Ø x37,100

Fig. 25 Anterior region, ventral surface of the complete hypoblast layer, O x37,100



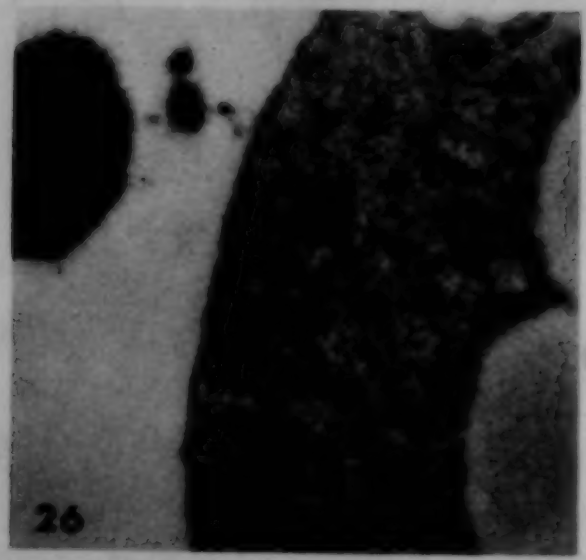
24



25

Fig. 26 Primitive streak region, dorsal surface of the epiblast in the primitive groove; ⌒ pattern, with no binding in the groove region. x37,100

Fig. 27 As in Fig. 26, but in the base of the primitive groove, ⌒ ← ; several epiblast cells are seen at the base of the primitive groove in the process of invaginating. x37,100



26

27

Fig. 28 Wall of the primitive groove, dorsal surface of the epiblast displays no discontinuity ⌒ with even staining ++ on entire surface, ++ x37,100

Fig. 29 As in Fig. 28, through base of the primitive groove, ⌒ ++, several epiblast cells are seen at the base of the primitive groove in the process of invaginating; D-cell debris. x37,100

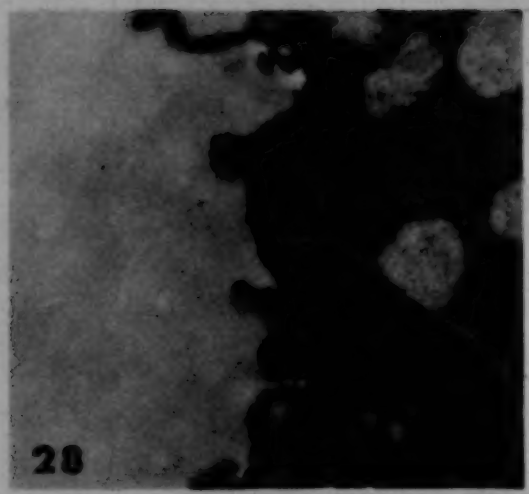
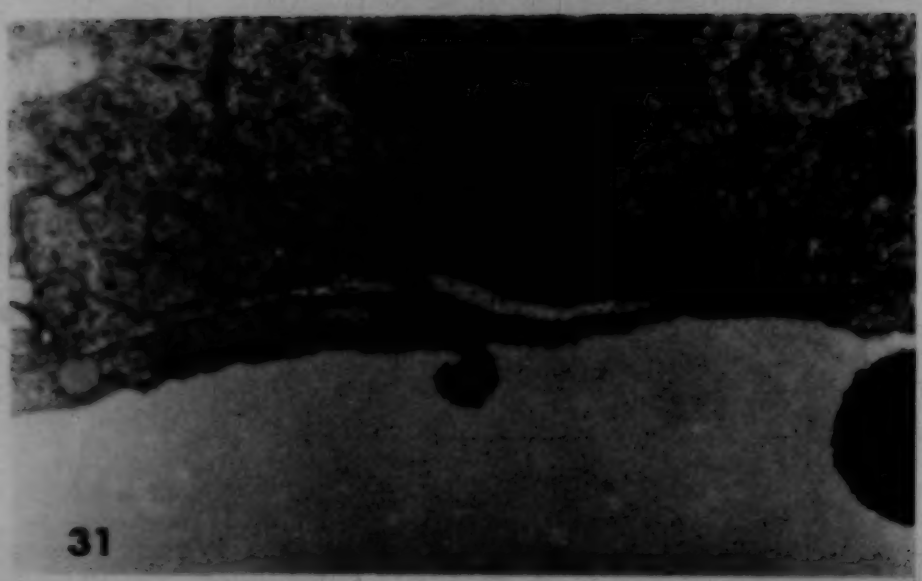

28

Fig. 30 Dorsal surface of epiblast in a region located towards the periphery of the embryo, i.e., lateral to the primitive streak, representative of both ⌐⌐ and ⌐⌐ patterns; see text, ++ x37,100

Fig. 31 Ventral surface of the hypoblast in sections of the primitive streak, representative of any part of the ventral hypoblast in both ⌐⌐ and ⌐⌐ patterns, 0 x18,200


31

Figs. 32 to 35 are representative of Con A-HRP-DAB reaction done in the presence of αMM - control embryos.

Fig. 32 Dorsal surface of the epiblast in the anterior region of stage 4-5; junctional complex. x37,100

Fig. 33 Ventral surface of the epiblast in the anterior region of stage 4-5; BL, basal lamina. x37,100

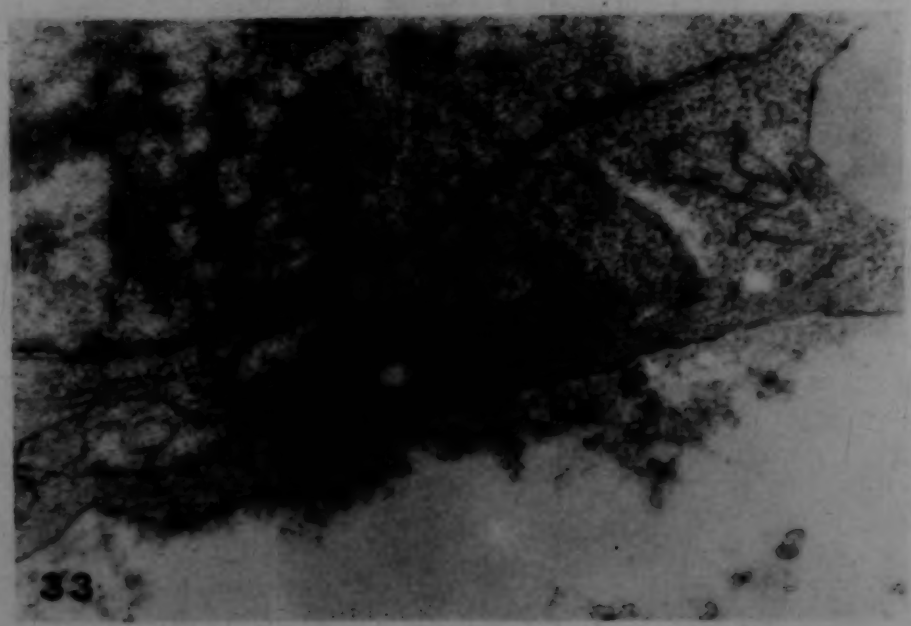
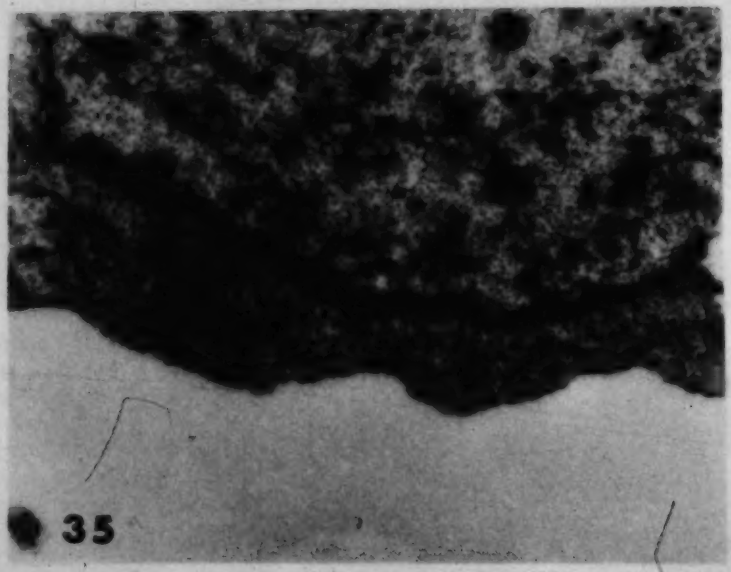

33

Fig. 34 Dorsal surface of the hypoblast in the anterior region of stage 4-5. x37,100

Fig. 35 Ventral surface of the hypoblast in the anterior region of stage 4-5. x37,100

34



35

Figs. 36 to 39, stage 1 embryos separated into epiblast fragments and treated with Con A-HRP-DAB.

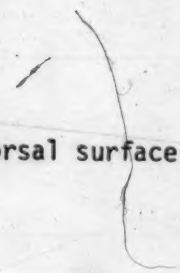
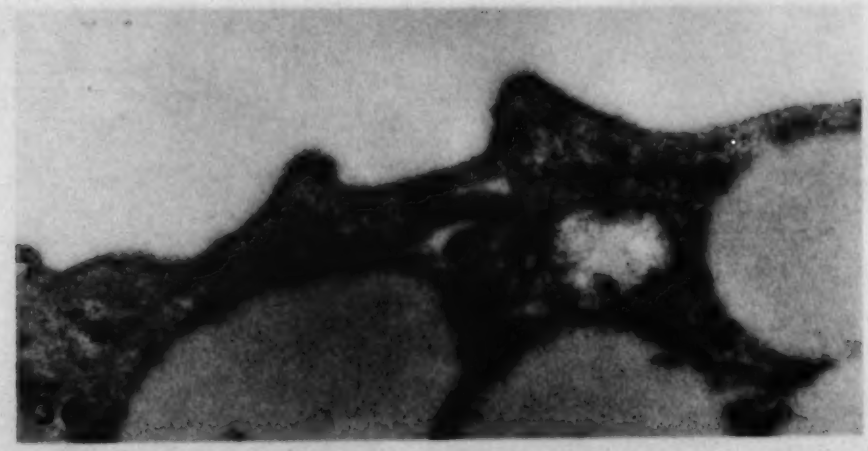
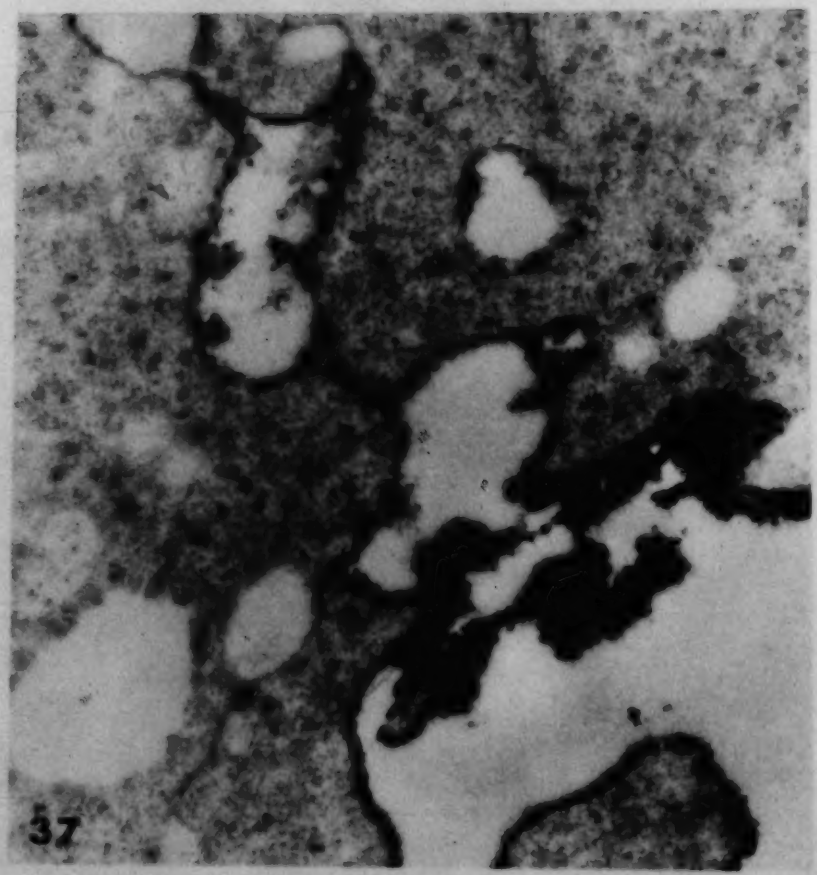


Fig. 36 Dorsal surface of epiblast, 0 x37,100

Fig. 37 Ventral surface of epiblast cells; reaction penetrates between two cells to stain the lateral surfaces; arrows indicate ventral surface; ++ x37,100

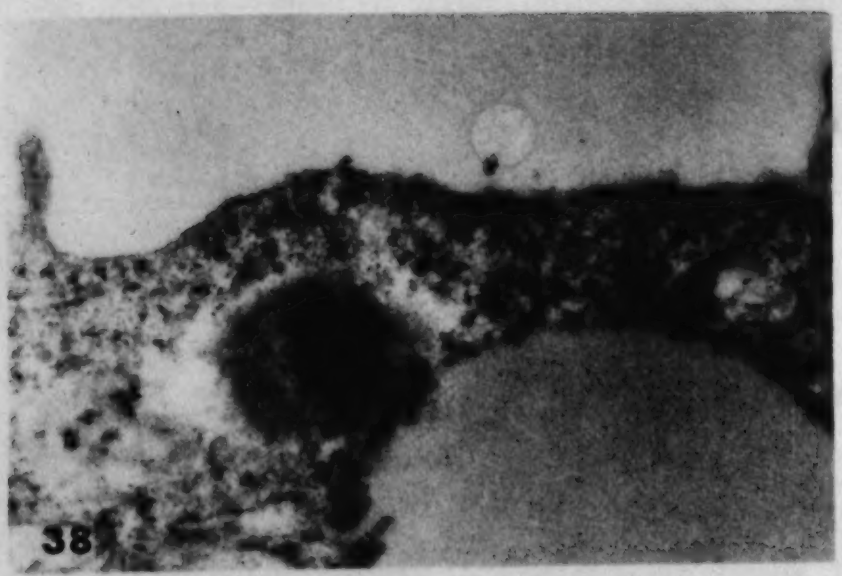



37

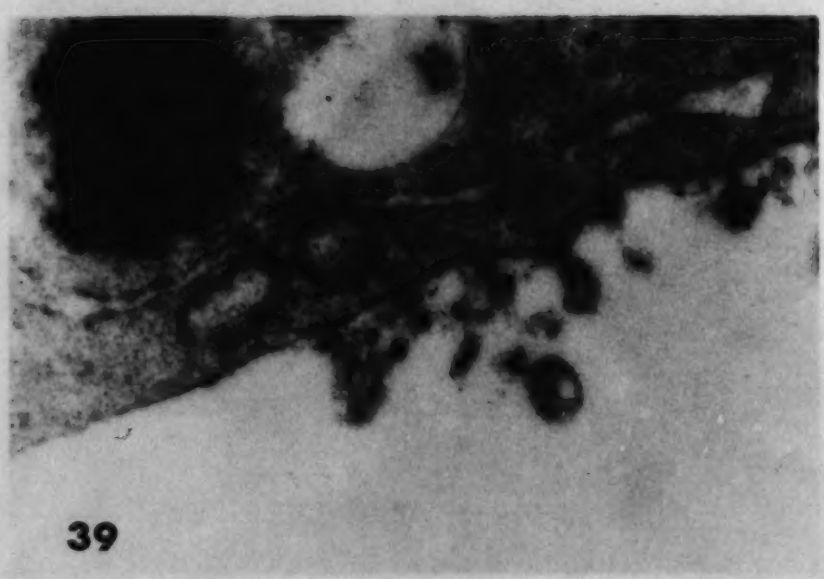
Figs. 38 and 39, stage 1 embryos separated into epiblast fragments and incubated with Con A-HRP-DAB and αMM; control embryos.

Fig. 38 Dorsal surface of the epiblast. x37,100

Fig. 39 Ventral surface of the epiblast. x37,100



38



39

3. *Ferritin-Con A*

This cytochemical procedure was performed on a total of nine stage 1 and twenty-two stage 4-5 embryos. Each ferritin molecule was seen as a particulate dense deposit, limited to the outer surface of the plasma membrane. Generally, these particles displayed a discontinuous pattern, appearing singly or in clumps separated by areas of membrane with no molecules bound. The amount of reaction product was scored by the amount of space between these clumps. A score of 0 (for example Fig. 51) was given if no reaction product was found, + (for example Fig. 53) if only a few clumps of ferritin were found widely spaced on the membrane, ++ (for example Fig. 40) if ferritin particles were separated by short distances, and +++ (for example Fig. 41) if all of the membrane was covered by ferritin particles.

None of the control embryos displayed any ferr-Con A binding in the presence of αMM (Figs. 59 and 60). In this respect controls with ferritin were much more consistent than controls with HRP-DAB. A blind experiment in which a total of twenty specimen grids were scored by an unbiased observed as either control or experimental material was run, and all nine control grids were correctly identified, as were all eleven experimental grids.

Ferr-Con A results are summarized in Table 2. Sections taken from anterior regions of stage 1 did not usually have a complete hypoblast, but there was no difference between anterior regions and posterior regions in the pattern of ferr-Con A binding, despite the difference in the completion of the hypoblast. The patterns shown by ferr-Con A did not display the same range of variability as found

Table 2

Summary of Results of Incubation With Ferritin-Concanavalin A

Stage 1			Stage 4-5		
	Anterior	Posterior		Anterior	Posterior
DE	++	++	DE	+++	+++
VE	+++	+++	VE	0 → + or Ø	0 → + or Ø
DH	+ → ++	+ → ++	DH	0 → + or Ø	0 → + or Ø
VH	+ → ++	+ → ++	VH	0 → +	0 → +

See text for evaluation procedure.

Abbrevations as in Table 1.

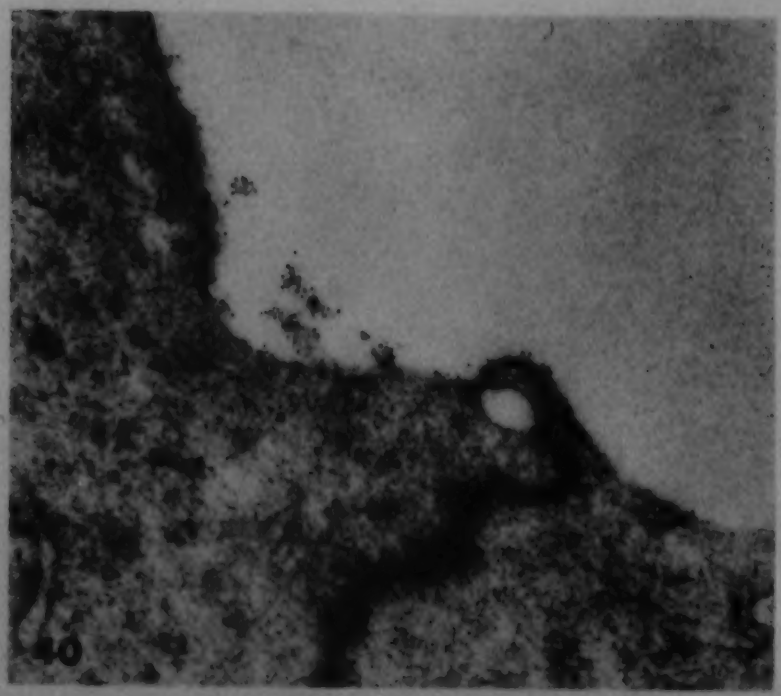
in Con A-HRP-DAB experiments. That is, there was greater consistency in the density of deposits on the corresponding surfaces of different embryos. The ventral surface of the epiblast showed a denser deposit (+++) (Figs. 41 and 46) than the dorsal surface of the epiblast (++) (Figs. 40 and 45). Single hypoblast cells showed a lighter deposit on a surface with no striking changes in binding intensity from dorsal to lateral to ventral surfaces (+ → ++) (Figs. 42, 43 and 44). In sections taken from posterior regions, no binding was found on lateral surfaces of hypoblast layer, but dorsal and ventral surfaces of the hypoblast had the same ferritin binding of similar intensity as in anterior regions (+ → ++) (Figs. 47 and 48).

Stage 4-5 embryos showed a heavier binding on the dorsal surface of the epiblast than that found on the dorsal surface of the epiblast in stage 1 (+++) (Figs. 50, 54, 55, 55 and 57). No differences in the binding were found in either anterior regions or areas through the primitive streak with respect to the dorsal surface of the epiblast. As well, the dorsal surface of the epiblast in regions of the primitive groove displayed no difference in binding with respect to areas more peripheral to the primitive streak. In both areas sectioned, the ventral surface of the epiblast (Fig. 51) and the dorsal surface of the hypoblast (Fig. 52) displayed virtually no reaction product except for a very few isolated clumps or no reaction product at all (0 → + or ∅). This variation may be due to the presence of undifferentiated junctions (Fig. 49) found in the hypoblast of stage 1, which may impede penetration to varying degrees. The ventral surface of the hypoblast in the anterior region (Fig. 53) and beneath the primitive streak

Figs. 40 to 60, Transmission electron micrographs of embryos treated with Ferritin-Con A. Figs. 40 to 49, stage 1 embryos. Figs. 50 to 58, stage 4-5 embryos.

Fig. 40 Anterior region of the blastoderm, dorsal surface of the epiblast, ++ x64,500

Fig. 41 Anterior region of the blastoderm, ventral surface of the epiblast, hypoblast incomplete layer, +++ x64,500



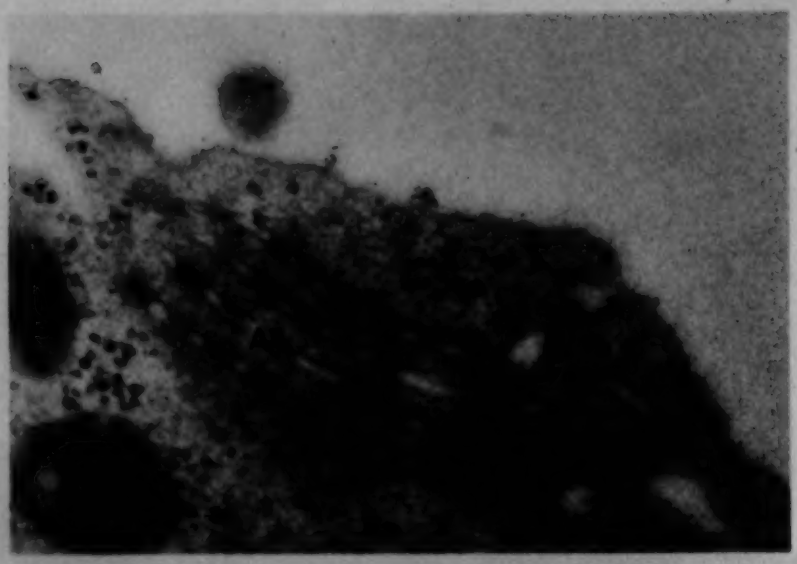
40

41

Fig. 42 Anterior region of the blastoderm, dorsal surface of the hypoblast cell; AL, annulate lamellae, + x64,500

Fig. 43 Anterior region of the blastoderm, lateral surface of the hypoblast cell, ++ x64,500

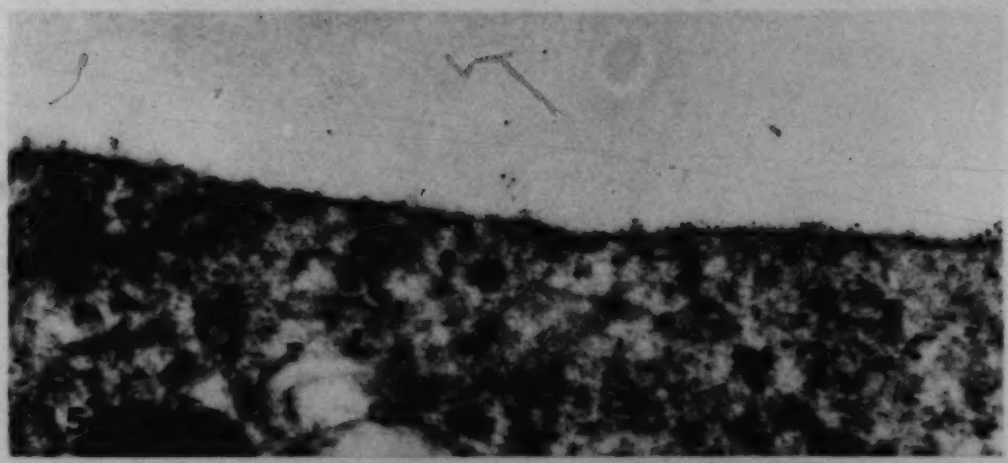
Fig. 44 Anterior region of the blastoderm, ventral surface of the hypoblast, ++ x64,500

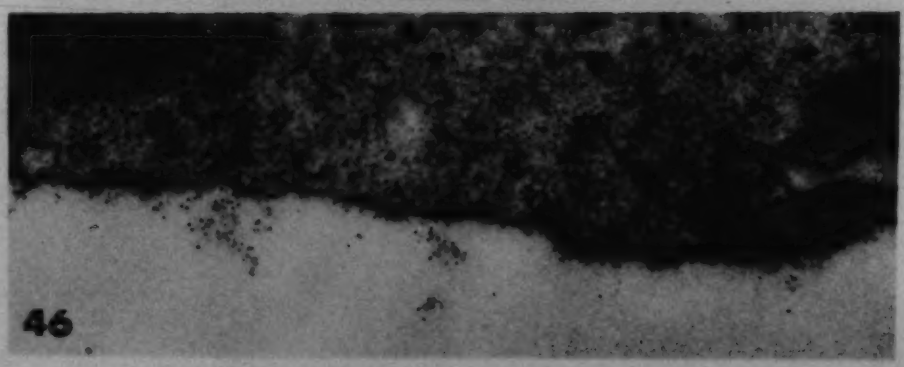


44

Fig. 45 Posterior region of the blastoderm, dorsal surface of the epiblast, ++ x64,500

Fig. 46 Posterior region of the blastoderm, ventral surface of the epiblast, +++; hypoblast complete layer. x64,500

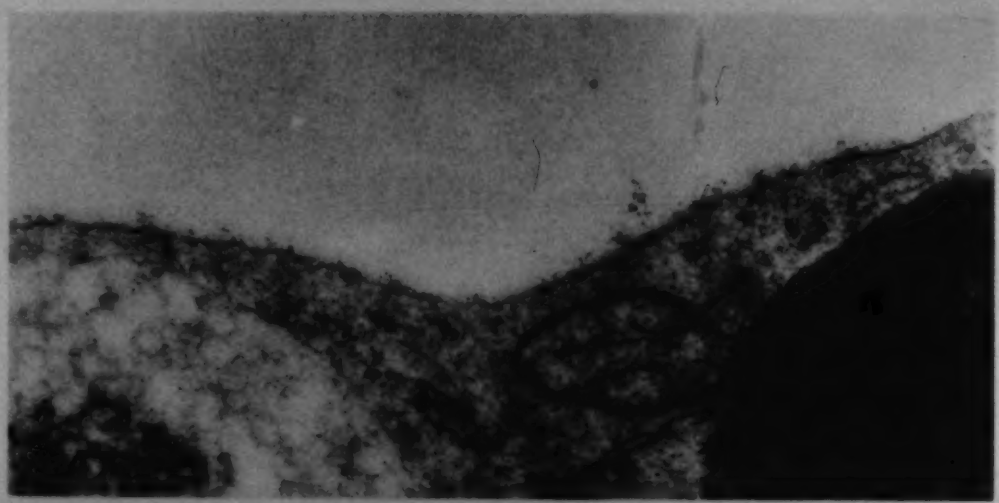




46

Fig. 47 Posterior region of the blastoderm, dorsal surface of the complete hypoblast layer, ++ x64,500

Fig. 48 Posterior region of the blastoderm, ventral surface of the complete hypoblast layer, ++ x64,500



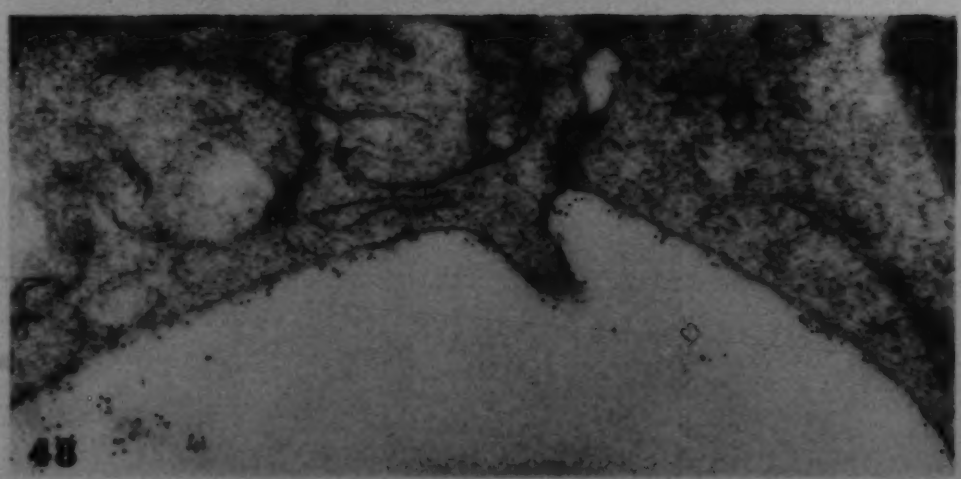
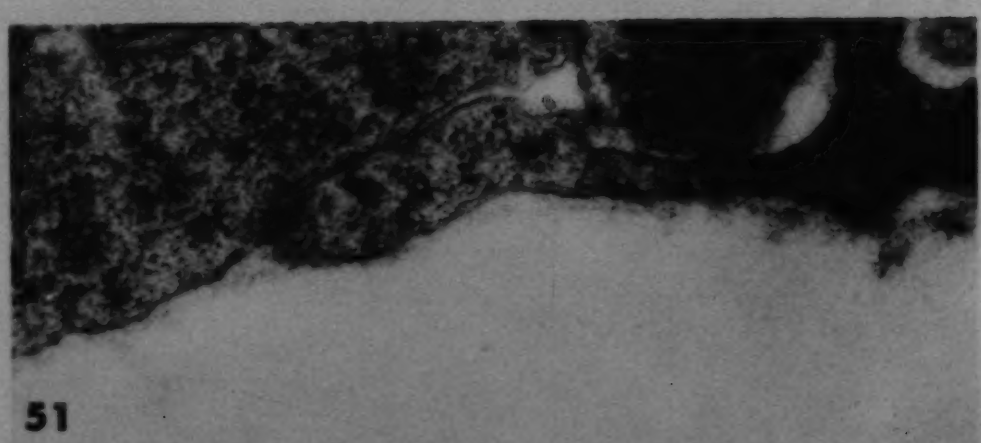

4B

Fig. 49 Undifferentiated junction found in a complete hypoblast layer of stage 1 embryo in the posterior region of the blastoderm. x77,900

Figs. 50 to 60, Stage 4-5 embryos treated with ferritin-Con A

Fig. 50 Anterior region of the blastoderm, dorsal surface of the epiblast, +++; C, cilium. x64,500

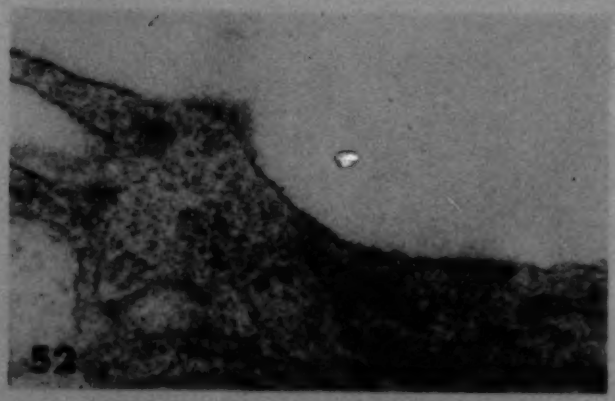
Fig. 51. Anterior region of the blastoderm, ventral surface of the epiblast, 0 or Ø, hypoblast is a complete layer. x64,500



51

Fig. 52 Anterior region of the blastoderm, dorsal surface of the complete hypoblast layer, O x64,500

Fig. 53 Anterior region of the blastoderm, ventral surface of the complete hypoblast layer, ++ x64,500

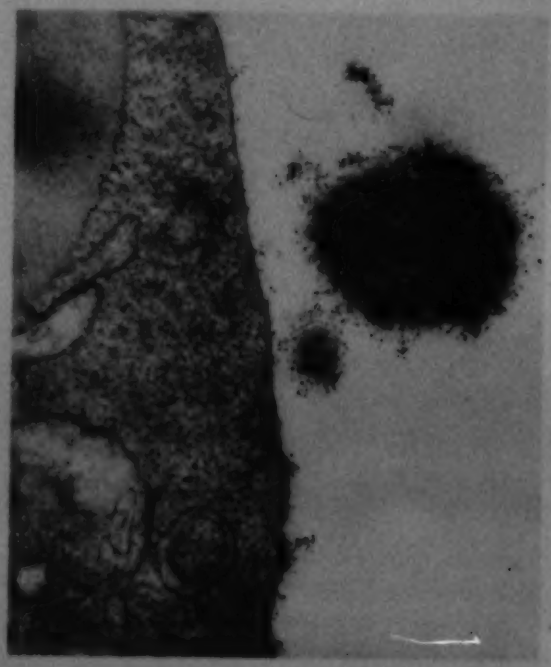


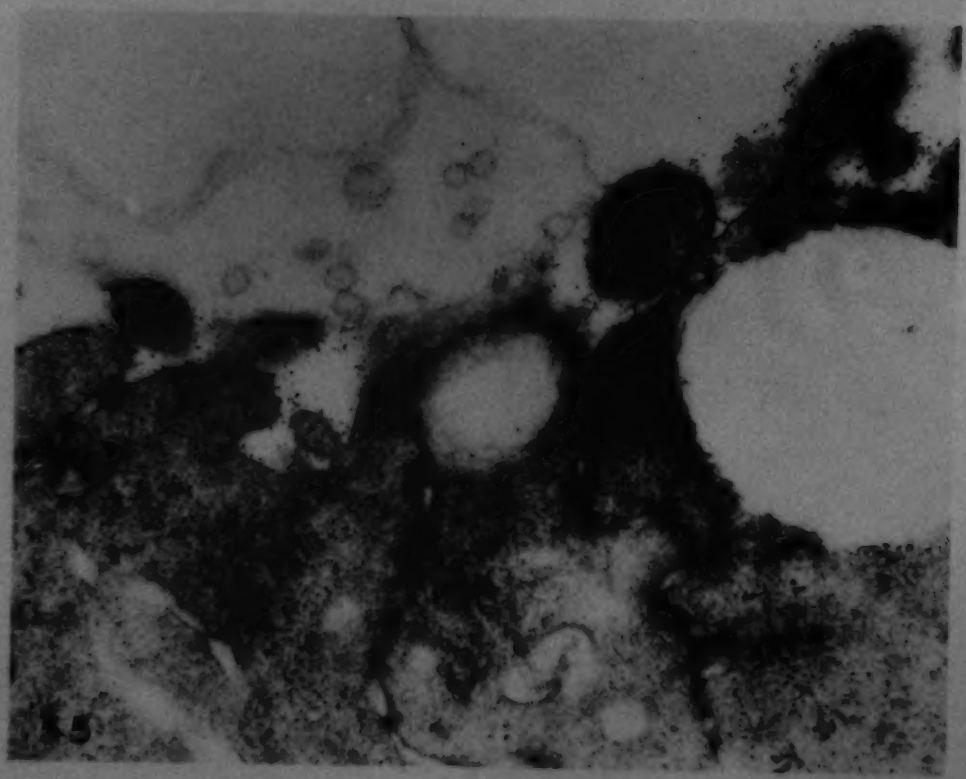
52

53

Fig. 54 Section through the primitive streak, dorsal surface of the epiblast in a primitive groove, +++ x64,500

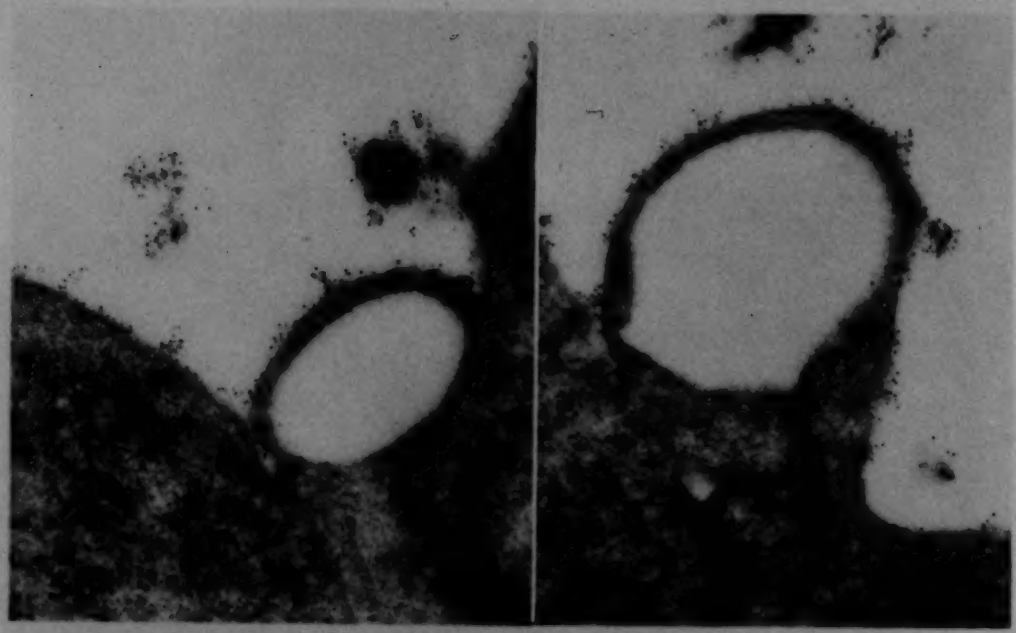
Fig. 55 As in Fig. 52, base of the primitive groove, +++; several epiblast cells are seen at the base of the primitive groove in the process of invaginating. x64,500





Figs. 56 and 57 Dorsal surface of the epiblast in a region located towards the periphery of the embryo, +++. CP, cell process. x64,500

Fig. 58 Ventral surface of the hypoblast in sections of the primitive streak; representative of any part of this surface in this region, + x64,500

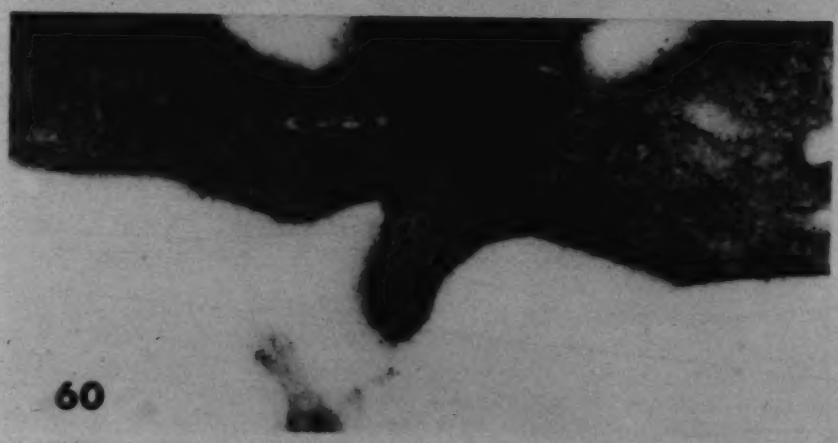


58

Figs. 59 and 60 are representative of Ferritin-Con A reaction done in the presence of αMM control embryos. x64,500

Fig. 59 Dorsal surface of the epiblast in the anterior region of the stage 4-5 embryo. x64,500

Fig. 60 Ventral surface of the hypoblast beneath the primitive streak region. x64,500



60

(Fig. 58) displayed a pattern with a few isolated clumps or no reaction product (0 → +). This region was variable in that three embryos out of eleven showed a denser staining (++).

4. *Iron Dextran-Concanavalin A*

A total of six stage 1 embryos was used with this cytochemical technique. Control embryos showed no binding and particles of electron dense material were scattered sparsely along surfaces of experimental embryos (Figs. 61 and 62). Very high magnification was required to view these particles and no further experiments were carried out with this technique since it was considered that evaluation would be unfeasible.

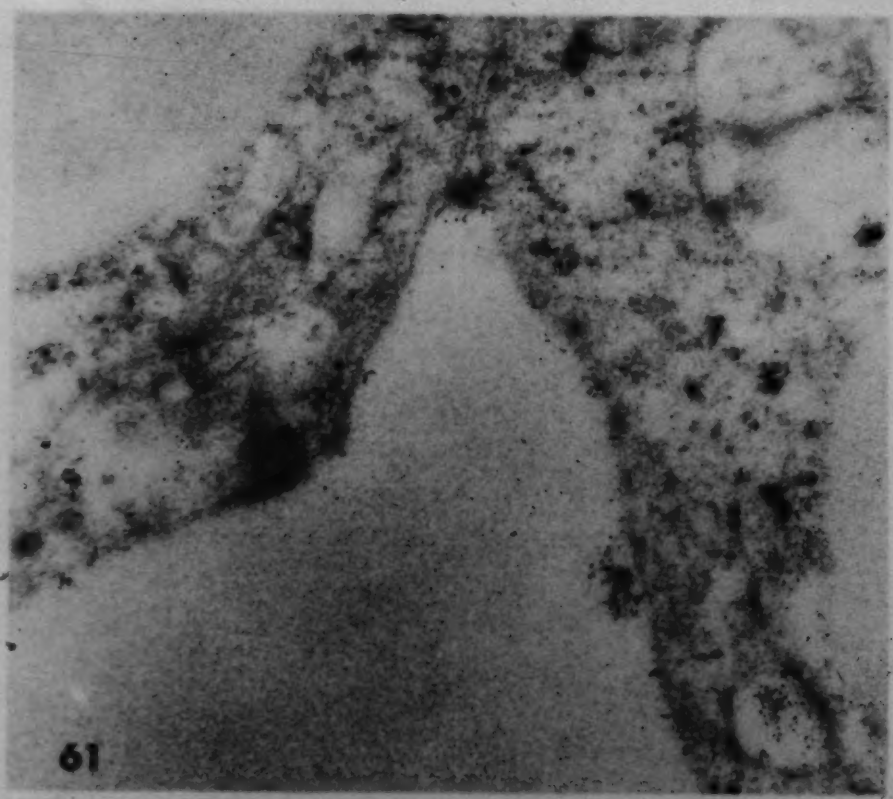
5. *FITC-Con A*

Eight stage 5 embryos were incubated in FITC-Con A. All control embryos showed a diffuse, non-specific staining over the entire embryo which could not be eliminated by increasing the concentration of hapten inhibitor or decreasing the incubation periods. The experimental and control embryos also displayed two parallel strips of densely stained tissue on either side of the primitive streak.

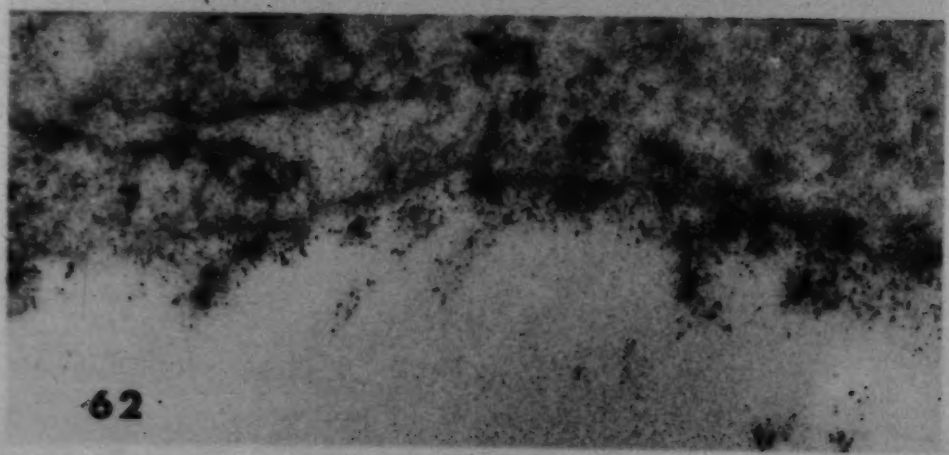
Figs. 61 and 62 are representative micrographs of stage 1 embryos incubated with iron dextran-Con A

Fig. 61 Ventral surface of the epiblast in the anterior region of the blastoderm. x77,900

Fig. 62. Ventral surface of the epiblast in the anterior region of the blastoderm. x77,900



61



62

DISCUSSION

The quaternary structure of the Con A molecule changes from dimer to tetramer under different pH and temperature conditions. As each protomer has one saccharide binding site, it is important to take into account the dimer-tetramer transition state of the molecule when evaluating binding sites on the cell surface. Huet *et al.* (1975) reported that decreased binding of radioactively-labelled cells resulted as temperature decreased from 37°C to 0°C and concomittently that the tetrameric form predominated at 37°C and dissociated into dimers as the temperature lowered. In this study, the cytochemical reactions were all carried out at 22°C and a pH of 6.8 to 7.2, conditions under which the Con A molecule would be predominantly in the tetramer configuration and therefore have four available saccharide binding sites (McKenzie *et al.*, 1972; Reeke *et al.*, 1975).

In this study, two cytochemical techniques were used which showed differences in the sensitivity for detecting CABS on the cell surface. The HRP-DAB technique is unquantitative in that local differences in the amount of cell-bound Con A are not always reflected by local differences in the reaction product deposited. There are several reasons for this. As the HRP molecule attaches to the cell-bound Con A molecule via its sugar residues, the lectin must have available free saccharide binding sites or that particular Con A molecule will not be detected by this technique; as well, it is theoretically possible for one lectin molecule to be labelled by more than one HRP molecule. Collard and Temmink (1974) also suggested that factors such as membrane

configuration or distribution of CABS may govern the amount of HRP-DAB reaction produced. For example, Con A bound very close together on the cell surface may not, for steric reasons, be able to bind HRP. Collard and Temmink (1974) also reported that HRP does not remove cell-bound Con A and concluded that the lectin has a greater affinity for membrane sites than for HRP.

Huet and Bernadac (1974) have also reported that the amount of Con A bound to the cell surface and the amount of HRP bound to lectin was not one-to-one. They found that only a few lectin molecules bound enzyme and the ratio varied between cell types. Huet and Bernadac (1974) suggested that structural modification of Con A may occur upon binding to sugar sites on the cell surface and alter the binding sites for the attachment of HRP. Others have reported conformational changes upon sugar binding. For example, Doyle *et al.* (1973) reported that ligand binding protected Con A against hydrolysis by pronase and against heat aggregation. If Con A is binding to different types of receptors, all of which contain the specific sugar, Huet and Bernadac argued that it was possible that each receptor may have different binding constants which in turn produce different structural rearrangements altering the Con A-HRP in a differential fashion.

Acting to offset these problems is the high activity of HRP at room temperature. Temmink *et al.* (1975) reported that even a few cell-bound HRP molecules yielded a saturated patch of DAB reaction product. This amplification process makes quantitative assessment of the number of binding sites more difficult for two reasons. Firstly, in a cluster of cell-bound HRP, if a few molecules react to produce a very dense reaction

product, then the expression of the remainder of the cell-bound HRP molecules cannot be seen. Secondly is the problem of subjective error; if a few molecules give a very dense deposit, it becomes difficult to assess the kind of reaction product between areas. That is, it is very easy to relate a medium-dense reaction (++) product to a greater number of binding sites than may actually be present.

These problems suggested that Con A labelled covalently *in vitro* with a cytochemical marker (ferritin) would be a better marker than Con A labelled *in situ* with a marker (HRP) via its sugar residues and enzymatically detected. However, Temmink *et al.* (1975) reported that even these types of markers do not detect 100% of the cell-bound Con A. These workers suggested that the covalent bond between the ferritin and Con A complex may make the complex less pliable and hence render some binding sites on the cell surface inaccessible. The relatively large size of the ferritin-Con A complex (approximately 120 Å) might prohibit the labelling of each CABS if receptors sites were packed very closely together. Huet and Garrido (1972) also suggested that as the covalent linkage between ferritin and Con A is stabilized by glutaraldehyde, this reaction with glutaraldehyde may make the Con A less reactive with binding sites on the cell surface. Ferritin-Con A, although not labelling 100% of the CABS sites available on a cell surface, does provide a more stoichiometric relationship between the number of reactive sites and the amount of ferritin visualized due to the particulate nature of the ferritin molecule.

Taking into account the differences between these two cytochemical techniques, remarkably little difference in the pattern of

binding was seen, despite the fact that two strains of hens were used. The biggest problem in these techniques was the inability to stoichiometrically relate binding of reaction product quantitatively to the number of CABS on the cell surface. The HRP-DAB technique was much more difficult to interpret in an objective fashion than the ferritin experiments due to the diffuse dense reaction product. This technique also seemed to be more susceptible to non-specific binding, as in each experiment a few controls would exhibit such binding. Others (for example Bernhard and Avrameas, 1971) have reported similar non-specific binding in the presence of the hapten inhibitor with this technique. Ferritin experiments, however, did not show this non-specific binding of marker in the presence of the hapten inhibitor. As well, comparing the experimental embryos, the variability between corresponding surfaces was not as great with the ferritin marker as with the HRP. This may reflect the greater sensitivity of HRP-DAB or the amplification process in which only a few molecules of bound HRP will produce a very dense reaction product.

Variability was greatest in the separation experiment in which the epiblast fragments taken from stage 1 embryos displayed patterns not found in intact embryos. For example, the dorsal surface of the epiblast always displayed reaction product in intact embryos in the areas sectioned. This may be a result of inadvertently separating pieces of epiblast from regions not normally sectioned in whole embryos. For example, it is conceivable that pieces of epiblast in extreme peripheral anterior or extreme posterior areas may be different from the medial anterior or medial central regions normally sectioned transversally in

whole embryos. Either serial transverse sections or techniques in which the whole dorsal surface of the epiblast could be examined, perhaps by scanning electron microscopy and the use of hemocyanin, could be used to investigate this possibility. As the cytochemical procedure was the same as that used with whole embryos, an alternative explanation may be that the separation procedure may in some way alter the membrane receptor sites.

Differences were seen between corresponding surfaces of stage 1 embryos with respect to cytochemical techniques. For example, the main discrepancy was that of the pattern seen on individual hypoblast cells with the HRP-DAB which, if present with ferritin, was not perceived. This pattern, of a very much denser reaction product on the ventral surface than on the dorsal surface, is probably the result of the very high activity of HRP and a consequent amplification effect which occurred during oxidation of the DAB. The other major difference was the penetration of ferritin between cells to label the ventral surface of the epiblast and the dorsal surface of the hypoblast when the hypoblast formed a coherent layer in the posterior regions of the embryo. The simpler cytochemical procedure, ferritin-Con A, in which labelling was dependent only on the penetration of one solution rather than three (as with Con A-HRP-DAB) seemed to label these surfaces more consistently than with the HRP-DAB technique. In this regard, these surfaces show similar binding characteristics in sections taken from regions with and without a coherent hypoblast when ferritin was used. Thus, intense (+++) ferritin binding of the ventral surface of the epiblast and light to medium (+ → ++) ferritin binding on the dorsal surface of the hypoblast

was in agreement with the binding shown on material stained with HRP-DAB in anterior regions where the hypoblast did not hinder penetration. Also in agreement was the medium (++) binding on the dorsal surface of the epiblast and the light to medium (+ → ++) binding on the ventral surface of the hypoblast. Neither of these surfaces was inaccessible to the cytochemical procedure.

Penetration of reaction product between cells at the ventral surface of the epiblast and the dorsal surface of the hypoblast was also a problem in stage 5 embryos. HRP-DAB did not penetrate to stain these surfaces but ferritin did so, labelling them, if at all, with a few scattered particles. This very light reaction may be due to the inaccessibility of these surfaces to even ferritin at this stage. In this connection, the appearance of an undifferentiated junction in the hypoblast found in a stage 1 embryo may be relevant. Such junctions in the hypoblast have not previously been reported and, in fact, this was the only one found in this study. However, if these incipient junctions are forming at these early stages, they may impede the penetration of either Con A and its marker molecules and this may account for the inability of these markers to penetrate through the hypoblast. Thus, information gathered in this study about these potentially inaccessible surfaces should be considered incomplete.

The dorsal surface of the epiblast in stage 5 was stained with a similar intensity with HRP in comparison to stage 1, but was more heavily stained with ferritin in comparison to stage 1. The two patterns (i.e., no labelling in the groove vs labelling in the groove) found with HRP in the region of the primitive streak were not found with

ferritin. That is, ferritin binding displayed a fairly heavy, even pattern with no discontinuity seen in the region of the primitive groove. In one experiment, the HRP-DAB reaction displayed a discontinuity with no binding of reaction product in the primitive groove and heavy binding on the epiblast immediately peripheral to the groove. This discrepancy might have resulted from staging differences, in which the differential affinity for the lectin occurred in the primitive groove at a critical point in development, or the group of embryos removed may have had different surface binding characteristics due to inherent seasonal differences, or the deposition of reaction product may have been affected by some undetected change in the lectin's affinity, perhaps due to storage. Efforts to repeat these experiments and find similar binding patterns were unsuccessful. The ventral surface of the hypoblast in both cytochemical procedures showed virtually no binding of reaction product.

In comparing the overall binding patterns expressed by both cytochemical techniques between stage 1 and stage 5, the most immediate difference was that of the staining pattern found on the ventral surface of the hypoblast. The reaction product disappeared from stage 1 to stage 5. Differences were also seen in the ventral surface of the epiblast. However, the abrupt change in stage 1 embryos from the very dense staining pattern on the ventral surface of the epiblast to no staining in stage 5 may also result from inaccessibility to even ferritin. As well, the reduction in binding to the dorsal surface of the hypoblast may also result from this inaccessibility of these surfaces.

During stage 1, a basement lamina (Fig. 33) is laid down on the ventral surface of the epiblast which can be seen as a diffuse amorphous

deposit (Martínez-Palomo, 1970). Martínez-Palomo reported that this structure was positive to ruthenium red at the primitive streak stage. Ruthenium red is specific for acid mucopolysaccharides. This study confirmed the presence of mannose and/or glucose residues in this structure as early as stage 1. Although no comparable staining of this structure occurred in stage 5, this may be due to penetration problems, although the disappearance or masking of these residues in the basement lamina by some other component cannot be ruled out by this study.

The heavier staining on the dorsal surface of the epiblast in stage 5 embryos with ferritin but not HRP-DAB may indicate the unmasking or production of more receptor sites on this surface in preparation for movement through the primitive streak as ferritin is a more stoichiometric method than HRP-DAB.

The most abrupt and clear-cut alteration in the binding patterns was with respect to the ventral surface of the hypoblast. In stage 1 this surface showed a positive reaction and in stage 5 this surface no longer showed a similar affinity for binding. This may reflect subpopulations present in the hypoblast. In stage 1 embryos, the hypoblast consists of cells invaginated from the epiblast and this layer forms as a gradual coalescence of these invaginated groups of cells (Eyal-Giladi, 1976). At stage 5, these cells have now moved in a peripheral fashion and have been replaced by the secondary hypoblast. It is this secondary contribution to the hypoblast which forms presumptive embryonic endoderm (Rosenquist, 1966). This study provided evidence that the hypoblast cells which are found beneath the primitive streak and which consists of cells from the primitive streak do not have receptor sites for Con A,

while the cells forming the primary hypoblast in stage 1 do have these receptor sites. As this secondary hypoblast results from the movement of cells from the epiblast, which also stained positively, the receptor sites are either masked, removed from the cell surface or made inaccessible to the lectin, perhaps by configurational changes in the receptors or by structural modifications of the cell surface.

In a study using immunofluorescent techniques it was found that the primary hypoblast cells displayed antigenic specificity (Wolk *et al.*, 1974). This antigenic specificity was used to trace the formation and migration of primary hypoblast. It was found that in unincubated stages all cells stained, but the lower part of the blastoderm stained with the most intensity, i.e., the ventral surface of the epiblast and a very intense staining on the hypoblast cells. A similar pattern was found in embryos with a complete hypoblast, but in primitive streak stages the fluorescence was heaviest in presumptive extra-embryonic endoderm cells; that is, hypoblast cells located in the lateral regions of the blastoderm. No fluorescence was found in cells in various embryonic layers, including the hypoblast underlying the primitive streak.

This study confirmed the differences in cell surface groups in the two populations of hypoblast cells, and it seems evident that this criterion may be used to indicate early signs of biochemical differentiation. At the same time, these receptor sites may be involved in processes controlling morphogenetic movement. For example, the distribution of sites which may be altered by cellular control via membrane-associated microtubules or microfilaments and the chemical composition of receptor sites can both be important factors relating to

selective adhesion and cellular movement. Or this could relate to cellular recognition or a form of cellular communication which allows groups of cells to move or not move in a coordinated fashion. Thus, these receptor sites could conceivably function in both senses to coordinate morphogenetic movement. It should also be pointed out that it is possible for regional specialization of cell surfaces to occur. For example, the dorsal and ventral surfaces of the epiblast are both located on the same cell and yet these two surfaces can display entirely different patterns of binding, perhaps in response to local conditions.

The current concept of cellular movement in the primitive streak is one of a coordinated movement of sheets of epiblast cells moving towards the primitive groove and then upon reaching the base of the groove, the movement of cells singly downward to join either the hypoblast or mesoblast and then subsequent lateral movement. The alterations in surface morphology would seem to suggest that the new structures seen are somehow involved in this process. The function of the cilia, found one per cell, and the loop-like processes found overlapping the epiblast cellular junctions on the dorsal surface, are unknown. The reason for the change from a cell surface enriched with microvilli to one with globular projections also has no known function. Bancroft and Bellairs (1974) correlated the appearance of microvilli with presumptive ectodermal covering of the embryo. These authors also reported that cells which are destined to invaginate possessed fewer microvilli but more globular projections. Although these globular projections may be associated with cell movement, Bard *et al.* (1975)

have suggested that they may be due to fixation artefacts. As well, the fate of the junctional complexes found in epiblast cells is unknown, but it would be expected that reorganization must occur as epiblast cells invaginate through the primitive streak.

This study provided evidence of a reduction in staining in cells which composed the hypoblast in stage 4-5 as compared to stage 1. Although cells dissociated from blastoderms of the same stages did not show any difference with respect to Con A-mediated agglutination (Zalik and Cook, 1976), this type of pattern may not be detected by agglutination studies for several reasons. Firstly, the dissociated blastoderms were not separated into epiblast, mesoblast and hypoblast populations. If these cell populations could be separated, each population may show a differential agglutinability. Secondly, the dissociation procedures may change the cell surface architecture and abolish any differences which were detected by the ultrastructural procedures used in this study.

The effects of preparing single-cell suspensions on the structure of the membrane and components involved in recognition and adhesion are unknown. McDonough and Lilien (1975a) reported that trypsin-dissociated cells were able to redistribute their CABS into caps with or without Con A, but if these cells were allowed to recover from trypsin treatment they lost this ability to redistribute receptor sites. Therefore, agglutination studies which employ trypsin dissociation procedures may be monitoring changes in the lateral mobility of receptor sites as much as the availability of these sites for agglutination.

Other studies (for example O'Dell *et al.*, 1974; Johnson, 1975; Roberson *et al.*, 1975) have used FITC-Con A to label receptor sites in

different developmental systems. Although caution must be exercised in comparing embryonic systems, a pattern has emerged. Generally, cells which are undergoing morphogenetic movement bind more FITC-Con A or display a greater lateral mobility of CABS in the plane of the membrane. A clustering of CABS into one area of the cell is taken to mean that that particular cell type exhibits a greater mobility of receptor sites than a cell with CABS randomly distributed over the cell surface. This capped appearance is abolished if the cells are fixed prior to exposure to Con A. For example, the migratory cell type in sea urchin embryos, the micromeres, were found to have a greater mobility of CABS than non-migrating cell types. Johnson (1975) reported that cells taken from dissociated gastrulas of *Rana* displayed a higher mobility of receptor sites than on dissociated blastula cells. In the present study, differences in lateral mobility of receptor sites were not seen, but the lectin was attached to fixed tissue and therefore this protocol would not detect such differences. O'Dell *et al.* (1974) studied the distribution of FITC-Con A over the surface of fixed *Xenopus* embryos. These authors concluded that the binding of FITC-Con A correlated with cells undergoing morphogenetic movement. Attempts to evaluate FITC-Con A binding on the cell surface of chick embryos in this study met with failure due to a high background of non-specific FITC-Con A binding. The low resolving power of the light microscope also limited the ability to detect differences in binding to particular cell populations. However, as most of the cells are undergoing migratory movements during primitive streak formation in the chick, it was shown by this study that all cells, except for those forming the secondary hypoblast, displayed binding.

Although these secondary hypoblast cells would be undergoing migratory movement, receptor sites other than those which bind Con A may become important in controlling the movement of these cells at this point in development.

This study provided evidence for alterations in surface morphology and chemical groups on the cell surface occurring from stage 1 to stage 5. Although Con A labels specifically the mannose and glucose residues plus sterically-related groups, the labelling found on the cell surface may not be specific in labelling different types or classes of receptors. If structural differences in the receptor sites were responsible for their respective functions, this labelling procedure could not be expected to detect these differences. The possibility of very subtle differences in receptors on the cell surface existing and not being detected by the lectin probe cannot be ruled out by this study. As well, these receptors may exhibit different binding affinities for the lectin, and these differences are not easily accessible in ultrastructural studies. The advantage of this study was in the use of receptors *in situ*, undisturbed by dissociation procedures or culturing techniques. By modifications of the procedure developed in this study, additional information regarding the mobility of the receptor sites as monitored by susceptibility for the Con A molecule to induce clustering in unfixed tissue could be gathered. It would be expected that if differences in relative mobility existed with respect to cell populations, then these cells would display their CABS in clumped or perhaps capped configurations, rather than the random distribution seen in this study. However, as it has been shown that cells within intact retina tissue

are not able to redistribute their receptors into caps (McDonough and Lilien, 1975a), this relationship between mobility of receptor sites and capped CABS in intact tissue may be difficult to interpret. As well, by employing the use of various enzymatic procedures, it may be possible to study the apparent disappearance of the CABS on the hypoblast in stage 5. The present study provided evidence for the existence of these receptors and showed an alteration in these structures with increasing embryonic age, but the function of these receptor sites remains to be elucidated.

BIBLIOGRAPHY

Avrameas, S. (1969) Immunochemistry *6*: 43-52.

Balinsky, B.I. and Walther, H. (1961) Acta embryol. morph. exp. *4*: 261-283.

Bancroft, M. and Bellairs, R. (1974) Cell Tiss. Res. *155*: 399-418

Bard, J.B.L., Hay, E.D. and Meller, S.M. (1975) Develop. Biol. *42*, 334-361.

Becker, J.W., Reeke, G.N., Cunningham, B.A. and Edelman, G.M. (1976) Nature *259*: 406-409.

Bellairs, R. (1971) *Developmental Processes in Higher Vertebrates*, Logos Press Ltd., London.

Bernhard, W. and Avrameas, S. (1971) Exptl. Cell Res. *64*: 232-236.

Bretscher, M.S. and Raff, M.C. (1975) Nature *258*: 43-49.

Brown, S. and Revel, J.P. (1976) J. Cell Biol. *68*: 629-641.

Collard, J.G. and Temmink, J.H.M. (1974) Exptl. Cell Res. *86*: 81-86.

Disse, J. (1878) Arch Mikr. Anat. *15*: 67-94.

Doyle, R.J., Nicholson, S.K. and Gray, T.D. (1973) Carbohyd. Res. *29*: 265-270.

Duval, M. (1884) Ann. Sci. Nat. Zool. VIe. Série T *18*: 1-208.

Edelman, G.M., Cunningham, B.A., Reeke, G.N., Becker, J.W., Waxdal, M.J. and Wang, J.L. (1972) Proc. Nat. Acad. Sci. U.S.A. *69*: 2580-2584.

Eyal-Giladi, H. (1970) J. embryol. exp. morph. *23*: 739-749.

Eyal-Giladi, H. and Wolk, M. (1970) Wilhelm Roux' Archiv. *165*: 226-241.

Eyal-Giladi, H., Farbiasz, I., Ostrovsky, D. and Hockman, J. (1975a) Develop. Biol. *45*: 358-365.

Eyal-Giladi, H., Kochav, S. and Yerushalmi, S. (1975b) Differentiation *4*: 57-60.

Eyal-Giladi, H. and Kochav, S. (1976) Develop. Biol. *49*: 321-337.

Finean, J.B., Coleman, R. and Michell, R.H. (1974) *Membranes and Their Cellular Functions*, Blackwell Scientific Publications, Oxford.

Goldstein, J.J., Reichert, C.M., Misaki, A. and Gorin, P.A.J. (1973) Biochim. Biophys. Acta *317*: 500-504.

Graham, R.C. and Karnovsky, M.J. (1966) J. Histochem. Cytochem. *14*: 291-302.

Hamburger, V. and Hamilton, H.L. (1951) J. Morph. *88*: 49-92.

Huet, C.H. and Garrido, J. (1972) Exptl. Cell Res. *75*: 523-527.

Huet, C.H. and Bernadac, A. (1974) Exptl. Cell Res. *89*: 429-431.

Huet, C.H., Lonchampt, M., Huet, M. and Bernadac, A. (1975) Biochim. Biophys. Acta *365*: 28-39.

Hughes, R.C. (1973) Prog. Biophys. Molec. Biol. *26*: 189-268.

Jacob, H.J., Christ, B., Jacob, M. and Byvank, G.J. (1974) Z. Anat. Entwickl-Gesch. *143*: 205-214.

Jacobson, W. (1938) J. Morph. *62*: 415-443.

Johnson, K.E. (1975) J. Cell Biol. *67*: 194(a).

Johnson, M.H., Eager, D. and Muggleton-Harris, A. (1975) Nature *257*: 321-322.

Kleinschuster, S.J. and Moscona, A.A. (1972) Exptl. Cell Res. *70*: 397-410.

Koller, C. (1882) Arch. Mikr. Anat. *20*: 174-211.

Krach, S.W., Green, A., Nicolson, G.L. and Oppenheimer, S.B. (1974) Exptl. Cell Res. *84*: 191-198.

Lallier, R. (1972) Exptl. Cell Res. *72*: 157-163.

Lee, H. (1976) Develop. Biol. *48*: 392-399.

Lis, H. and Sharon, N. (1973) Ann. Rev. Biochem. *42*: 541-574.

Macarak, E.J. (1975) J. Morph. *147*: 41-60.

Martin, B.J. and Spicer, S.S. (1974) J. Histochem. Cytochem. *22*: 206-209.

Martínez-Palomo, A. (1970) Int. Rev. Cytol. *29*: 29-75.

McDonough, J. and Lilien, J. (1975a) J. Cell Sci. *19*: 357-368.

McDonough, J. and Lilien, J. (1975b) Nature *256*: 416-417.

McKenzie, G.H., Sawyer, W.H. and Nichol, L.W. (1972) Biochim. Biophys. Acta *263*: 283-293.

Merback, H. (1935) Z. Anat. Entwickl-Gesch. *104*: 635-652.

Meissier, P. and Auclair, C. (1974) Develop. Biol. *36*: 218-223.

Moran, D. (1974a) J. Exp. Zool. *188*: 361-366.

Moran, D. (1974b) Exptl. Cell Res. *86*: 365-373.

Moscona, A.A. (1968) Develop. Biol. *18*: 250-277.

Moscona, A.A. (1971) Science *171*: 905-907.

Moscona, A.A. (1974) in *The Cell Surface in Development*, ed. Moscona, A.A. New York, Wiley.

Nicolson, G.L. (1972) Nature, New Biol. *239*: 193-197.

Nicolson, G.L. (1974) Int. Rev. Cytol. *39*: 89-190.

Nicolson, G.L. (1976) Biochim. Biophys. Acta *457*: 57-108.

Nicolson, G.L. and Singer, S.J. (1971) Proc. Nat. Acad. Sci. U.S.A. *68*: 942-945.

Nicolson, G.L. and Singer, S.J. (1974) J. Cell Biol. *60*: 236-248.

O'Dell, D.S. (1972) Cytobios. *6*: 91-96.

O'Dell, D.S., Ortolani, G. and Monroy, A. (1973) Exptl. Cell Res. *83*: 408-411.

O'Dell, D.S., Tencer, R., Monroy, A. and Brachet, J. (1974) Cell Differentiation *3*: 193-198.

Peter, K. (1938) Z. Mikr. Anat. Forsch. *43*: 362-415.

Reeke, G.N., Becker, J.W., Cunningham, B.A., Wang, J.L., Yahara, I. and Edelman, G.M. (1975) Adv. Exptl. Med. Biol. *55*: 13-33.

Roberson, M., Neri, A. and Oppenheimer, S.B. (1975) Science *189*: 639-640.

Roberson, M. and Oppenheimer, S.B. (1975) Exptl. Cell Res. *91*: 263-268.

Roseman, S. (1970) Chem. Phys. Lipids *5*: 270-297.

Rosenquist, G.C. (1966) Contr. Embryol. Carneg. Inst. *38*: 71-113.

Rosenquist, G.C. (1971) Develop. Biol. *26*: 323-335.

Roth, S. (1973) Quart. Rev. Biol. *48*: 541-563.

Roth, S., McGuire, E.J. and Roseman, S. (1971) J. Cell Biol. *51*: 536-547.

Ruggeri, A. (1966) Arch. ital. Anat. Embriol. *71*: 407-432.

Sanders, E.J. and Zalik, S.E. (1972) J. Cell. Physiol. *79*: 235-247.

Sanders, E.J. and Zalik, S.E. (1976) Differentiation *6*: 1-11.

Sharon, N. and Lis, H. (1975) in *Methods in Membrane Biology*, ed. E. Korn. Plenum Press, New York.

Shur, B. and Roth, S. (1973) Amer. Zool. *13*: 1129-1135.

Shur, B.D. and Roth, S. (1975) Biochim. Biophys. Acta *415*: 473-512.

Singer, S.J. and Nicolson, G.L. (1972) Science *175*: 720-731.

Smith, C.W. and Hollers, J.C. (1970) J. Reticuloendothel. Soc. *8*: 458-464.

Smith, S.B. and Revel, J.P. (1972) Develop. Biol. *27*: 434-441.

Spratt, N.T. and Haas, H. (1960) J. Exp. Zool. *144*: 257-275.

Spratt, N.T. and Haas, H. (1965) J. Exp. Zool. *158*: 9-38.

Temmink, J.H.M., Collard, J.G., Spits, H. and Roos, E. (1975) Exptl. Cell Res. *92*: 307-322.

Tyler, A. (1946) Growth *10*: 7-19.

Uchida, T. and Matsumoto, T. (1972) Biochim. Biophys. Acta *257*: 230-234.

Vacquier, V.D. and O'Dell, D.S. (1975) Exptl. Cell Res. *90*: 465-468.

Vakaet, L. (1962) J. Embryol. Exp. Morph. *10*: 38-57.

Vakaet, L. (1970) Arch. Biol. (Liège) *81*: 387-426.

Weeks, G. (1973) Exptl. Cell Res. *76*: 467-470.

Weeks, C. and Weeks, G. (1975) Exptl. Cell Res. *92*: 372-382.

Weinbaum, G. and Burger, M.M. (1973) Nature *244*: 510-512.

Weiser, M.M. (1972) Science *177*: 525-526.

Weiss, L. (1967) *The Cell Periphery, Metastasis and Other Contact Phenomena*, New York, Wiley.

Weiss, P. (1947) Yale J. Biol. Med. *19*: 235-278.

Weller, N.K. (1974) J. Cell Biol. *63*: 699-707.

Wenzler, R.J. (1970) Int. Rev. Cytol. *29*: 77-125.

Wolk, M., Schlesinger, M. and Eyal-Giladi, H. (1973) Isr. J. Zool. *23*: 204-205.

Yariv, J., Kalb, A.J. and Levitake, I.A. (1968) Biochim. Biophys. Acta *165*: 303-305.

Zalik, S.E., Sanders, E.J. and Tilley, C. (1972) J. Cell. Physiol. *79*: 225-234.

Zalik, S.E. and Sanders, E.J. (1974) Differentiation *2*: 25-28.

Zalik, S.E. and Cook, G.M.W. (1976) Biochim. Biophys. Acta *419*: 119-136.

Zwilling, E. (1963) Develop. Biol. *7*: 642-652.